DETECTION AND IDENTIFICATION OF PLANT VIRUSES BY IMMUNO-ELECTRON MICROSCOPY

by

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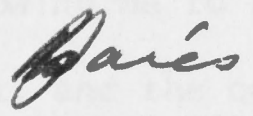
This Thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, it contains no material previously published or the result of work by another person, except where due reference is made in the text.



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"It is doubted whether a man ever brings his faculties to bear with their full force on a subject until he writes upon it."

Cicero.

Dedicated to Penny.

SUMMARY

Immuno-electron microscopy (IEM) was first developed 6 years before this project was commenced. In this time there have been many reported variations but no comparisons have been made of the relative sensitivities of the range of variations. I have compared the range of methods under the same conditions with the same viruses from the Tobamovirus group and my results have established that the slower methods are necessary for maximum sensitivity of virus detection and that the key factor that governs the sensitivity is the virus acquisition time. I have demonstrated that the time used to coat grids with antiserum is of no importance providing it is at least 10 minutes.

The procedures of IEM seemed to have a good potential for increasing particle numbers on a grid, consequently minimising the number of micrographs required for measurements of virus particles, with a resulting considerable saving in time. While this is so for isometric plant viruses, I have shown in Section 4 that the method results in an unexpectedly high proportion of small particles in the population present on the grids when rod-shaped Tobamovirus particles are used. I consider that my results amply demonstrate that the method is not suitable for use in measuring rod-shaped virus particles.

In the same section I have pointed out some anomalies in the published population size distributions of some Tobamoviruses and the standard parameters used to measure the size of virus particles in a population.

I have then examined the method of IEM when used for identification of viruses trapped on a grid. To obtain comparative figures I have utilised a measure which I have called an "affinity ratio" that

gives an indication of the closeness or otherwise of serological relationships as determined by IEM. Two methods used to identify viruses by IEM are counts of trapped particles and another known as "clumping". I have demonstrated in Section 5 that particle counts are not very reliable and that it is greatly affected by the virus concentration in the plant sample used. It has been reported in the literature that clumping also has the same deficiency.

The way to use IEM most effectively for identification is to be completely independent of the starting concentration of virus and this is achieved by the method that is known as "decoration". This is very useful for determining virus identity. I have developed a useful modification of this method which I have called "gold-labelled antibody decoration" (GLAD). A preliminary report of this method and its application has been accepted for publication.

I have then applied these methods to a field situation, in which samples vary in virus concentration by large amounts, to determine the relative values of the variations in the IEM technique and to obtain information on the Tobamoviruses present in orchids and tomatoes in New South Wales. This has shown clearly that decoration and GLAD are the most useful techniques to identify viruses by IEM and has shown the advantages and disadvantages of the methods used in a field situation. This is the first published account of the use of IEM in a field survey, and in the light of the results I have suggested what would be a more suitable way to carry out such a survey.

KEY TO ABBREVIATIONS

Abbreviations are explained when first used in the text but many of the more commonly used ones are listed here for easier reference.

1. Viruses, Antisera and Gold Conjugated Antisera

U1-TMV Type (U1) strain of tobacco mosaic virus.

U2-TMV U2 strain of tobacco mosaic virus.

ToMV Tomato mosaic virus.

ORSV Odontoglossum ringspot virus.

SHMV Sunn-hemp mosaic virus.

CV4 CV4 strain of tobacco mosaic virus.

When the virus abbreviation is preceded by "a." it refers to the antiserum to this virus. When preceded by "G/a." it refers to the antiserum conjugated to gold-protein A.

NS - Normal serum.

2. Other

AM - Ammonium molybdate.

CB - Carbonate buffer.

EM - Electron microscope.

GLAD - Gold-labelled antibody decoration.

IEM - Immuno-electron microscopy.

IgG - Immuno gamma globulin.

MW - Molecular weight.

pA - protein-A

PB - Phosphate buffer.

PBS - Phosphate buffered saline.

RNA - Ribo-nucleic acid.

SSC - Saline sodium citrate.

VAT - Virus acquisition time.

CONTENTS

		Page No.
1.	INTRODUCTION: PLANT VIRUSES, THEIR DETECTION AND IDENTIFICATION	6.
	1.1. Introduction	7.
	1.1.1. *Definition of a virus.*	7.
	1.1.2. *Appearance and grouping.*	7.
	1.1.3. *Detection and diagnosis.*	8.
	1.2. Virus-specific protein	11.
	1.2.1. *Virus-specific, non-coat protein.*	11.
	1.2.2. *Virus coat protein.*	12.
	1.3. Serology	16.
	1.4. Nucleic acids	19.
	1.4.1. *General.*	19.
	1.4.2. *Methodology of nucleic acid research.*	20.
	1.5. Immuno-electron microscopy	27.
2.	MATERIALS AND METHODS	37.
	2.1. Viruses	38.
	2.1.1. *Maintenance of isolates.*	38.
	2.1.2. *Terminology.*	39.
	2.1.3. *Serological relationships.*	39.
	2.1.4. *Purification.*	39.
	2.2. Antisera	40.
	2.2.1. *Preparation and storage.*	40.
	2.2.2. *Terminology.*	41.
	2.3. Grid preparation and electron microscopy	41.
	2.3.1. *Normal negative staining.*	41.
	2.3.2. *Immuno-electron microscopy.*	42.
	2.3.3. *Electron microscopy.*	43.
	2.4. The GLAD technique	44.
	2.4.1. *Preparation of colloidal gold-protein A.*	44.
	2.4.2. *Preparation and use of gold-antisera.*	45.

	Page No.
2.5. Buffers and solutions	46.
2.5.1. *Phosphate buffer.*	46.
2.5.2. *Carbonate buffer.*	46.
2.5.3. *Ammonium molybdate.*	46.
2.5.4. *Saline sodium citrate.*	46.
3. COMPARISON OF IEM METHODS	47.
3.1. Introduction	48.
3.2. Comparison of methods	49.
3.2.1. *Materials and Methods.*	49.
3.2.2. *Results.*	50.
3.2.3. *Discussion.*	51.
3.3. An examination of some of the factors involved in the relative sensitivities of the rapid and slow methods	51.
3.3.1. *Experimental.*	52.
3.3.2. *Discussion.*	54.
3.4. General Discussion	56.
3.4.1. *Concluding remarks.*	63.
4. THE EFFECT OF IEM ON PARTICLE SIZE DISTRIBUTION OF TOBAMOVIRUSES	66.
4.1. Introduction	67.
4.2. Materials and Methods	68.
4.3. The effect of trapping on particle size distribution	70.
4.3.1. *Materials and Methods.*	70.
4.3.2. *Results.*	71.
4.3.3. *Discussion.*	72.
4.4. The effect of different IEM methods	73.
4.5. The effect of sample freezing and IEM	75.
4.6. Comparison of populations after purification and after IEM	77.

Page No.

4.7. The use of protective agents to overcome changes after IEM 79.

4.8. General Discussion 81.

4.8.1. *Published sizes of Tobamoviruses.* 81.

4.8.2. *Particle size distributions of ORSV and SHMV.* 82

4.8.3. *The significance of small particles.* 84.

4.8.4. *Factors affecting the size of virus particles.* 86.

4.8.5. *Stability of Tobamoviruses.* 87.

4.8.6. *Effects of IEM on Tobamoviruses.* 89.

4.8.7. *The origin of small particles in trapped populations.* 90.

4.8.8. *Concluding remarks.* 92.

5. COMPARATIVE COUNTS OF TRAPPED VIRUS PARTICLES 95.

5.1. Introduction 96.

5.2. Antiserum dilution and diluents 96.

5.2.1. *Comparison of SSC, NS and CB as antiserum diluents.* 98.

5.2.2. *Homologous and heterologous trapping using higher concentrations of SSC.* 102.

5.3. Coating and virus acquisition times 105.

5.3.1. *Effect of short and long coating and virus acquisition times on virus identification.* 106.

5.3.2. *The effect of coating and virus acquisition times on the number of trapped particles.* 111.

5.3.3. *Discussion.* 114.

5.4. Precoating grids with protein-A 119.

5.4.1. *Protein-A and antiserum dilutions.* 120.

5.5. Interaction of virus dilution and the number of trapped particles 123.

5.5.1. *Purified virus suspensions.* 123.

5.5.2. *Sap extracts from infected plants.* 124.

5.5.3. *Discussion.* 125.

5.6. Identification of viruses by trapping with different antisera 128.

	Page No.
5.7. General Discussion	131.
5.7.1. *Coating antiserum.*	131.
5.7.2. *Virus preparation.*	135.
5.7.3. *Diagnostic use of particle trapping.*	135.
6. GOLD-LABELLED ANTIBODY DECORATION (GLAD)	139.
6.1. Introduction	140.
6.2. Decoration & label	145.
6.3. GLAD	147.
6.3.1. *Preliminary experimental work.*	147.
6.3.2. *Use of GLAD in Tobamovirus comparisons.*	151.
(a) *Distant relationships.*	151
(b) *Closer related viruses.*	154.
(c) *Comparison of 3 viruses and their antisera.*	156.
6.3.3. *GLAD and Tymoviruses.*	157.
6.4. General Discussion	159.
7. APPLICATION OF IEM TO FIELD SURVEYING OF VIRUS INCIDENCE	162.
7.1. Introduction	163.
7.2. Orchids	165.
7.2.1. *Field survey.*	166.
7.2.2. *I.E.M.*	167.
7.3. Tomatoes	170.
7.3.1. *Survey of glasshouse crops.*	170.
7.3.2. *Comparison of 5 isolates.*	172.
7.3.3. *Isolates 69A and 69B.*	174.
7.3.4. *Isolate 5.*	176.
7.3.5. *Discussion.*	177.
7.4. General Discussion	178.
8. OVERALL DISCUSSION	185.
8.1. The usual method of virus detection and identification	187.

Page No.

8.2. The IEM method 188.

8.2.1. *Virus detection by IEM.* 188.

8.2.2. *Virus identification by IEM.* 192.

8.2.3. *IEM and virus measurement.* 195.

8.3. Tobamoviruses of orchids and tomatoes 196.

8.4. Concluding comments 197.

9. REFERENCES CITED 199.

FIGURES

SECTION 4.

Fig.	After p.
4/1.	68.
4/2.	70.
4/3.	71.
4/4.	74.
4/5.	76.
4/6.	76.
4/7.	78.
4/8.	84.
4/9.	85.

SECTION 5.

Fig.	After p.
5/1.	97.
5/2.	99.
5/3.	100.
5/4.	100.
5/5.	103.
5/6.	111.
5/7.	113.
5/8.	121.
5/9.	121.
5/10.	124.
5/11.	125.
5/12.	125.

SECTION 6.

Plate 6/1. -- After p. 148.

1. INTRODUCTION

PLANT VIRUSES, THEIR DETECTION AND DIAGNOSIS:

A LITERATURE SURVEY

"To be perfectly original one should think much and read little, and this is impossible, for one must have read before one has learnt to think."

Byron.

1.1. Introduction to Plant Viruses, their detection and diagnosis

1.1.1. *Definition.*

There have been many definitions of viruses, all of which have certain advantages and disadvantages. The following can be used as a guideline for the basic properties of a virus and is quoted from Matthews (1970):

"A set of one or more nucleic acid template molecules, either DNA or RNA with the following properties:

1) They have the ability to organise their own reduplication only in a suitable intracellular environment. Intracellular components upon which virus replication is dependent include ribosomes, transfer RNA's and an energy producing system. Certain viruses may also require the presence of another virus.

2) In the mature virus particle, the genetic material is usually encased in a protective coat of protein or lipoprotein. Where the genetic material consists of more than one nucleic acid molecule, each may be housed in a separate particle or all may be housed in one. The mature virus contains no nucleic acids other than the genetic material.

3) A virus can cause disease, at least on one host, under appropriate conditions."

While conveying the idea that a virus consists only of a nucleic acid and a protein or lipoprotein it does not give any idea of general appearance, size, etc. Because of this some general introduction to plant viruses seems in place.

1.1.2. *Appearance and Grouping.*

Plant viruses can occur in one of 3 general shapes:

(i) Anisometric: Rod-shaped, either rigid or flexuous.

(ii) Isometric: generally approximately "spherical", appearing circular or hexagonal in outline in the electron microscope. Many are icosahedra.

(iii) Rhabdoviruses: Bullet-shaped or bacilliform.

Groups (ii) and (iii) do not readily lend themselves to grouping by morphology as there is not sufficient variation in size and shape when negatively stained or shadowed preparations are examined in the electron microscope (EM).

Group (i), however, has a large variation in appearance and size and can be grouped according to size and shape and the groups formed in this way also show some serological relationships between members of a group (Brandes and Bercks, 1965; Brandes and Wetter, 1959). Later these groupings were essentially maintained and some isometric viruses grouped according to features other than morphology (Harrison et al., 1971).

1.1.3. *Detection and Diagnosis.*

There is currently a range of techniques and characters used to detect, diagnose and classify plant viruses. These methods also vary greatly in their accuracy and ability to detect very closely related viruses:

(i) Symptoms. Use is made both of symptoms produced on the original host and those produced by the virus on infection of a somewhat "standard" host range. With viruses that can be readily sap ("mechanically") transmitted this is a fairly easy task but requires a lot of space in suitably temperature-controlled glasshouses. Some viruses are not mechanically transmissible and have to be transmitted by their appropriate insect vectors (if they are known) and this can entail a lot more time and expertise.

Symptoms can be useful in surveying crops for the presence or absence of viruses, surveys for a particular virus and in most instances for the differentiation of strains.

(ii) Electron Microscopy. Use of the EM has proved invaluable in virology laboratories and can be used in preliminary screening of samples suspected of being virus infected. The EM is of particular use for rod-shaped viruses where size can be estimated or measured and the virus thereby placed into a group. Often knowledge of the host plant may then serve to enable a diagnosis to be made. The technique is of much less use when the viruses are isometric, as some are broken up during preparation and many cannot be distinguished from constituents of plant cells commonly seen in negatively stained sap preparations (e.g. Beier and Shepherd, 1978).

(iii) Serology. This important technique of plant virology will be discussed in more detail in a separate section (1.3.) of this review but a few points will be commented on here.

There are many varied uses of serology in the science of plant virology and in discussing these Gibbs and Harrison (1976) considered the following to be the most important:

. To index plants for infection, as for example in the large-scale commercial testing of "seed" potato plants or tubers for potato viruses S or X.

. To determine the degree of antigenic relationship between the particles of different viruses or isolates.

. To determine the amount of virus nucleoprotein or protein in a preparation.

. To locate virus antigens in tissues or cells.

. To provide information on the structure of virus particles.

Van Regenmortel (1978) considers that the diagnosis of viruses by serology has continued to supplant inoculation to indicator hosts and that serology can detect viruses at much lower levels of concentration than the use of other techniques.

In spite of these statements serology has one disadvantage that, because of the specificity of the reaction, some idea of the virus identity has to be ascertained, prior to undertaking the serological tests, in order to reduce the serological testing to reasonably practicable levels. In surveying crops of vegetatively propagated material for virus infection the technique of serology is only of use when specific viruses are being tested.

At present, however, serology is an invaluable tool for classification of viruses and establishing criteria for identification of serological strains. One problem with strain categorisation and detection is that some strains differ from each other by small amounts and the end-points of reactions of two strains may differ by only one or two dilutions, which is within the errors due to the biological variations of the technique (e.g. differences between individual rabbits).

(iv) Immuno-electron microscopy (I.E.M.). This technique will be discussed in more detail in section 1.5. It is also known as serologically specific electron microscopy, and combines some of the advantages of visualisation with the specificity of serology. There are a number of variations in methodology culminating, to date, in the combination of specific-antibody coated grids and "decoration" of particles. The use of these methods also requires some knowledge of the viruses being examined before the grids can be coated with the appropriate antibody or the correct "decorating" antiserum applied.

The separation of strains is difficult by IEM and usually necessitates counting virus particles trapped on coated grids and in the case of very closely related serological strains this method is probably of little or no use unless some methods of very specific decoration can be developed.

(v) Physicochemical Characteristics. Included under this heading are features such as longevity in vitro, dilution end point, a thermal inactivation point, buoyant density etc. There is usually not much variation between viruses within a group and these properties are therefore of most use for allocating a virus to a particular group.

1.2. Virus-Specific Protein

The sequence of nucleotides on the nucleic acid of viruses contains the information that codes for the production of proteins in infected cells. The nucleic acid of tobacco mosaic virus (TMV), which is about 6,400 nucleotides long, codes for the coat protein and 4-8 other proteins. The coat protein is easily recognisable and relatively easy to isolate along with the nucleic acid in the form of virus nucleoprotein. As a result the coat has become the most studied virus-specific protein and very little is known about the others for most plant viruses.

1.2.1. *Virus-specific, non-coat protein.*

Very little information has been obtained about this class of proteins produced by plant viruses although some attempt has been made to examine them in association with animal viruses (e.g. Influenza virus - Gregoriades, 1977).

As they constitute the end-products of the majority of information on the virus genome they should be worthy of more study

and may show greater differences for strains than do coat proteins.

One type of virus-specific, non-coat protein that has been subjected to a good deal of study is that which forms proteinaceous inclusions induced by infections of Potyviruses. They have been reviewed in detail by Edwardson (1974). Studies have mainly been confined to electron microscopy and some serology but no detailed analyses of amino-acids or peptides have been made.

These inclusions have been classified into 3 morphological types (called pinwheels, tubes and laminated aggregates) and, based on the types present in an infected cell, the appearance and the relative abundance of each, some classification can be made of the viruses within the Potyvirus group and in some instances can serve to diagnose a particular virus.

The relatively small amount of serology done on these proteins has been reviewed (Israel and Wilson, 1977), the main points being that the inclusions are not serologically related to the virus coat proteins or the host proteins and there does not appear to be any serological difference between inclusions produced by different strains of the one virus.

1.2.2. *Virus Coat Protein.*

Being the most obvious and most easily isolated virus-induced protein, most studies have been carried out upon the coat protein. Not only have detailed analyses been done, but coat protein is, of course, responsible for the serological reactions on which large-scale research has been carried out and which will be discussed in a separate section of this review.

Special methods have been developed for obtaining purified coat protein free from nucleic acid (Ralph and Bergquist, 1967; Matthews, 1970). Once a protein has been purified it can be split up into polypeptides and then into amino acids, each of which can be studied and analysed to provide further information.

a) Studies on Whole Protein.

Apart from utilisation of surface antigenic properties most studies on undegraded protein utilise a range of techniques designed to separate one protein from another by the variations in molecular weight, surface charges, a combination of these two (e.g. polyacrylamide gel electrophoresis - PAGE) or isoelectric point (by including a pH gradient in the separation technique). Various combinations can be used for 2-dimensional analysis, as has been done, for example, with poliovirus proteins (Hamann et al., 1977).

The small differences that occur in the composition of proteins found in the strains of a virus are not sufficient to cause appreciable differences in the features used for analysis. In some instances the M.W. can distinguish between viruses that are in the same virus group (Veerisetty and Brakke, 1977).

Generally these techniques, when used to analyse whole protein, are best suited to establishing the broad groupings of viruses.

b) Peptides.

The differences between closely related proteins is due to small variations in the number and arrangement of the amino acids and this can be reflected in the peptides produced by enzymatic digestion of proteins. Most of the techniques that are used for proteins can be used for peptides (e.g. PAGE).

One method that has proved of great value in analysing peptides is peptide mapping or "fingerprinting". Essentially it consists in separating the peptides by a combination of chromatography and electrophoresis either on paper or thin-layer supports. The technique can be used for analytical mapping or preparative purification of peptides.

Konigsberg and Steinman (1977) consider that peptide mapping is reliable in extreme cases of completely identical or totally different polypeptide chains but that uncertainty may arise when an intermediate number of peptides is counted. They consider that such counting could be due to differences between the chains or due to 2 complications: (a) the inability to resolve all the peptides (which can be overcome by additional chromatographic electrophoretic separations); (b) lack of specificity or incompleteness of proteolytic cleavage (often an inescapable consequence of peculiarities of the polypeptide sequence).

Tung and Knight (1972) used peptide mapping on paper to look at some strains of TMV. They concluded that the accuracy of amino acid analysis/peptide mapping was affected by such factors as homogeneity of the protein, completeness of the tryptic digestion, overlapping of spots on the maps (which increased greatly above 15-20 spots) and the composition of the protein. They considered that the composition of the peptides can be determined without question and the summation gives the MW. Further they were of the opinion that the most straightforward and accurate procedure for determining the MW of homologous virus proteins is the combination of peptide mapping and amino acid analysis.

Faed et al. (1972) worked with small amounts of the minor proteins of turnip yellow mosaic virus (TYMV) and so used a thin-layer

2-dimensional mapping procedure which enabled the detection of up to 27 peptides. Only 16 were found when TYMV was analysed on paper. They assumed that the additional peptides were from non-specific tryptic cleavage and were present in amounts too small to be detected by the paper mapping system.

Peptide analysis is sensitive enough to show differences between strains and between viruses within a group, often agreeing with serological analyses.

c) Amino Acids.

Peptides can be further dissociated into amino acids that can be analysed and sequenced. Many viruses and strains of them have been subjected to amino acid analysis but not many have been completely sequenced.

There is nothing unusual about the amino acid composition of plant viruses - they are made up of the same 20 amino acids found in all other proteins. Matthews (1970) considered that the amino acid compositions showed a general trend in that (a) cysteine methionine, tryptophan, histidine and tyrosine usually occur in low amounts and (b) Hydroxy amino acids (serine and threonine) are relatively high.

Depending on the viruses studied there have been variations in the degree of correlation between amino acid compositions and serology.

Using strains of TYMV and other viruses Symons et al. (1963) found that the protein varied between viruses but not between strains. Similarly Moghal and Francki (1976) compared the amino acids of 6 different Potyviruses and found a correlation between amino acid

analysis and serology. There was no detailed examination of any strains of these viruses.

Somewhat contrary to this Wang and Knight (1967) found some strain variation when they examined the proteins of 13 strains of TMV isolated from tomatoes throughout the world. They could determine a relationship between strains in amino acid composition, C-terminal amino acids (which differed from type-TMV), and general amino acid sequence as determined by peptide mapping. Of the 13 strains examined 5 had an amino acid composition equivalent to tomato atypical mosaic, 7 had 1 exchange and 1 had 2 exchanges. Serologically there was the suggestion of a common epitope in the tomato strains that was distinct from any in TMV.

Later a study was made of orchid strains of TMV (Kado, et al., 1968). They examined 7 isolates and found 4 were serologically indistinguishable from common TMV but were distinct from each other and TMV when amino acids were compared. They were also distinguishable from each other by symptom expression on herbaceous hosts. The Odontoglossum ringspot (ORSV) isolates, instead of differing by a few amino acids from TMV, differed by about 15 amino acid exchanges and shared antigenic determinants with the HR and U2 strains of TMV.

1.3. Serology

Serology has developed into a field of scientific study by itself and has applications in many areas of biological research other than virology. Many tests have been developed over the years to increase specificity and detection end-points or to meet specialised requirements. To give some idea of the diversity of serological tests the following list is given based on Bercks, et al. (1972):

In liquid:	Precipitin tests in tubes
	Precipitin tests on slides
	Microprecipitin tests
	Ring interface precipitation
	Chloroplast agglutination
	Sheep red blood cell precipitation
In semi solid media:	Single diffusion
	Double diffusion (in one and two dimensions)
	Immunoelectrophoresis
	Immuno-osmophoresis
Absorption tests:	Passive haemagglutination
	Haemagglutination inhibition
	Bentonite flocculation
	Latex

Labelled antibodies

Complement fixation

Immune adherence

Neutralisation of infectivity.

These authors caution that it should be kept in mind that in studies of serological relationships a biological method is applied which is subject to all the laws of biological variation. Because of these variables van Regenmortel (1966) considered that there were many reasons for not relying solely on serological data for classification. Van Regenmortel (1975) studied strains of TMV and found difficulties in quantifying the extent of cross-reactivity due to variations from individual animals, the route and number of injections used and the time of bleeding.

The specialised terminology used in serology is defined clearly by Jerne (1960) and the molecular mechanisms of the antibody-

antigen complex have been discussed at length (e.g. Feinstein and Rowe, 1965; Almeida and Waterson, 1969).

The coat proteins of a virus are of course responsible for the serological reactions but it has been noted (Cowan, 1973) that conformational factors may play a vital role in determining the antigenic characteristics of several viruses. As an instance of this he quotes that, using TYMV and TMV, inoculations with virions produced much higher levels of precipitating antibody than if equivalent amounts of empty capsids were used. The addition of RNA did not increase the immuno-genicity of empty capsids. He was mainly concerned with the primary response of the inoculated animals.

The applications and uses of serology in the field of plant virology have been reviewed by van Regenmortel (1966, 1978).

Many serological techniques have been developed for work in fields other than plant virology and as the basic techniques (e.g. sampling tissues) in plant virology differ from those used in animal virology many methods have had to be tested on plant viruses and modified where necessary and possible (e.g. Abu Salih et al., 1968a, b; Bercks and Querfuth, 1971). Some plant virus proteins are not stable enough to act as immunogens without stabilisation by a fixative (Francki and Habili, 1972).

One very useful method for examining the serological relationships between viruses is the use of double diffusion in an agar gel. Precipitation lines form where the two reagents occur in their optimal ratios and the shape and confluence of the reaction lines can give a guide to the relationship between the various antigens. Unlike animal viruses, many plant viruses are long rods and those

longer than 300 nm do not pass through the gel. A number of reagents have been tested which satisfactorily fragment the rods and do not give false serological relationships (Purciful and Shepherd, 1964).

1.4. Nucleic Acids

1.4.1. *General.*

The genomes of plant viruses may be single stranded RNA (ssRNA), double stranded RNA (dsRNA) or dsDNA. The genome of the majority of plant viruses is a linear piece of ssRNA.

All nucleic acids consist of a repeating backbone of alternating sugars and phosphate residues with a purine or pyrimidine base attached to each sugar. In DNA the sugar is deoxyribose, the purines are guanine and adenine, and the pyrimidines are cytosine and thymine. In RNA the sugar is ribose, the purines are guanine and adenine, and the pyrimidines are cytosine and uracil.

In dsRNA and dsDNA the purine bases on one strand are paired with the pyrimidine bases on the other strand and when the nucleic acids are analysed:

guanine = cytosine (dsRNA and dsDNA)
adenine = uracil (dsRNA)
adenine = thymine (dsDNA)

There is no such pairing of bases with ssRNA.

When they summarised some characteristics of some well known plant viruses, Gibbs and Harrison (1976) noted that the molecular weight of most ssRNA in plant viruses was in the order of 2×10^6 but the MW's range from 0.4×10^6 (satellite virus) to 15.5×10^6 (clover wound tumour virus).

The genomes of plant viruses mostly occur in one piece but in some viruses they are divided into 2 or more parts, and when this

occurs one or more pieces (depending on the virus) may be necessary for the virus to infect a host and produce its "usual" range of symptoms and particle types.

Information contained in the sequence of bases in a nucleic acid is translated in the host cell into amino acids which are then combined to form proteins.

I will now briefly examine the various ways in which nucleic acids can be studied, with emphasis on plant virus ssRNA's which can be prepared to a greater degree of purity than can whole virus particles.

1.4.2. *Methodology of Nucleic Acid Research.*

Vital to the study of viral nucleic acids is the preparation of pure nucleic acid from purified virus preparations without further breakdown from enzymatic impurities. Virus RNA was first separated from entire virus particles by phenol extraction of TMV (Gierer and Schramm, 1956). Since then many techniques have been developed for use in different circumstances, and these have been collated and documented by Ralph and Bergquist (1967) and Matthews (1970).

Ralph and Bergquist (1967) offer 9 generalisations about these methods which are worth listing here: (i) 8-hydroxyquinoline can help to chelate contaminant metal ions; (ii) analytical reagent grades of phenol are suitable for this type of work; (iii) addition of nuclease inhibitors (e.g. SDS, polyvinyl sulphate or bentonite) is helpful; (iv) solubility of phenol in aqueous solution is a function of ionic strength and temperature (25°C is considered to be a suitable temperature); (v) high salt concentrations (>0.1M) can reduce the yield; (vi) at high virus protein concentrations the viral RNA can become trapped in the gel of protein that accumulates at the

phenol/water interface - this is overcome by using adequate water and phenol phases; (vii) many contaminating substances co-precipitate with RNA after ethanol addition; (viii) they suggest a number of methods for further purification of RNA; (ix) the phenol method can fail to produce infectious preparations from some viruses.

a) Base Composition. Essentially these studies require separation of the RNA from the virus, hydrolysis of the RNA, separation of the nucleotides, and measurement of the bases, usually by spectrophotometry (each base has a characteristic absorption spectrum). One method of analysing TMV-RNA is detailed by Whitfeld and Higgins (1976).

When the base compositions are plotted as a histogram and the overall patterns are compared (Gibbs and Harrison, 1976) it can be seen that related viruses have a similar appearance.

Earlier Gibbs (1969) compared the published base ratios of plant viruses, estimated the similarities by 3 methods and then classified them using 2 sorting strategies. Using these methods the plant viruses sorted into 6 groups. Two other points that came out of the study were that (i) Independent estimates on the same virus or strains of a virus usually classify together and (ii) Estimates for different fractions of one virus usually classified together in spite of the fact that with some fractions the gene products of the nucleic acids have different functions.

However in more recent studies of the cowpea strain of TMV (Whitfeld and Higgins, 1976) it was noted that there were two main particle sizes: short (40 nm long) and long (300 nm) and that the base ratios of the two varied - the short RNA had less adenylic acid and more uridylic acid than the long RNA and the purine:pyrimidine

ratio was 1.04 for the short and 1.20 for the long RNA. It may be that the cowpea strain of TMV is one of the exceptions to the findings of Gibbs (1969) or that Whitfeld and Higgins were looking for smaller, valid differences than would be necessary for the overall comparative study of Gibbs.

b) Oligonucleotide Composition. Some enzymes can hydrolyse RNA at specific linkages and the resultant fragments can be separated, identified and quantified. Early investigations of TMV-RNA showed that the base composition of intact nucleic acids (Knight, 1952; Cooper and Loring, 1954) and that of pancreatic ribonuclease resistant residues prepared from them (Reddi and Knight, 1956) were essentially the same in all strains examined.

Later an examination of monopyrimidine nucleotides was carried out (Reddi, 1957) and it was found that there were significantly greater amounts of cytidylic and uridylic acids in the digest of strain M than in TMV, HR and YA. Reddi (1959) then examined the di- and tri-nucleotides of TMV, HR and M and found differences between the strains. As the base ratios were the same for all strains the differences must have been due to the arrangement of bases along the nucleic acid.

Rushizky and Knight (1960) then developed a method for splitting TMV.RNA into oligonucleotides and mapping them. They used this technique to examine common TMV with a population of 75% or more (by wt.) of the characteristic 300 nm long rods. About 25% of the D_{260nm} substance fractionated was recovered in the form of an insoluble residue and 65% as mono and oligonucleotides.

The mapping procedure showed some advantages over other techniques (e.g. ion exchange and column chromatography) which yield

a few more fractions - it is convenient and requires a smaller amount of RNA. The authors concluded that the method showed promise for comparisons of strain nucleic acids as well as nucleic acids from different viruses. The increasing availability of ribonucleases of different specificities also adds another dimension to the possibilities of this technique.

Symons et al. (1963) examined the RNA of 6 strains of TYMV and found they fell into 2 groups - one had 38.2 molar % cytosine and one had 41.6%. Mono-, di-, and tri-nucleotides showed marked differences between these 2 groups and small, statistically significant differences within groups. Parallel studies on the proteins showed amino acid differences between groups but not within groups.

c) Base Sequencing. All proteins specified by a virus are determined by the coding on the RNA molecule, and the coding is due to the sequence of bases along the nucleic acid molecule. Thus any difference between viruses or strains of viruses must also be accompanied by a change in the sequence of bases, even though this change may only be 1 or 2 bases in some instances. There are 2 approaches to comparing the base sequences of viruses; one is to determine the actual sequence on each virus, and the other is to compare the similarities of the sequences of 2 viruses without knowing the exact sequence of either (sequence homology studies).

(i) Sequence Determination. The genome RNA of TMV is approximately 6,400 nucleotides and as there are no unusual bases to act as markers the problem of sequencing the entire molecule becomes almost impossible. Sequencing is attempted by breaking the molecule in different places by different chemicals and then determining the sequences in the pieces, "then with judgement, luck and the help of

an anagram enthusiast, the base sequence is derived" (Gibbs and Harrison, 1976). This has proved such a formidable task that the sequence has not yet been determined for any plant virus although attempts have been made culminating recently (Guilley et al., 1979) in the reported sequencing of 1,000 nucleotides at the 3' end of TMV-RNA.

(ii) Base Sequence Homologies. To date there have been two principal methods developed for molecular hybridisation studies of base sequence homologies, one using dsRNA formed in infected plant tissues and one using ds complementary DNA produced in vitro.

(a) Use of dsRNA. The general basis of these studies is that dsRNA is isolated from infected tissues, as are ^{3}H labelled- and unlabelled-viral RNA. Under appropriate conditions dsRNA, ^{3}H.RNA (homologous to the dsRNA) and the viral RNA to be tested, are heated to melting temperature, cooled and then incubated so that the single strands from the dsRNA combine with the viral RNA under test or the ^{3}H.RNA. These ds hybrids are then resistant to degradation by pancreatic ribonuclease and the more radioactivity that can be detected in the enzyme-resistant portions the less relationship between the test RNA and the ^{3}H.RNA.

One of the earlier uses of this technique was in a study of about 15 strains of TMV obtained from around the world (Vandewalle and Siegel, 1976). The strains fell into several groups with the nucleotide sequences being indistinguishable within a group and without similarity between groups. The same groups were seen based on capsid protein studies. Two groups, U1 and Dahlemense, had most alike capsid protein and yet the nucleic acids showed no homology.

Molecular hybridisation using complementary RNA (cRNA) has been used in a range of studies including gene functions of fowl plague

virus RNA, in which the technique is described in some detail (Scholtissek, et al., 1976), the homology of alphaviruses (Wengler et al., 1977) and the study of viroids using cRNA produced by the use of Qβ replicase (Owens and Diener, 1977).

Zaitlin et al., (1977) examined 3 strains of TMV using competition molecular hybridisation and found that strain C_T was homologous with U1 and neither showed any detectable relationship to strain C_C even though all three strains have many physical, chemical and biological properties in common and all showed a similar replication strategy.

Using RNA-RNA hybridisation to study the origins of human influenza subtypes, Scholtissek et al., (1978) found that the error in the method was in the order of 5%.

(b) Use of Complementary DNA (c-DNA). A more recent development of molecular hybridisation studies on virus RNA involves the use of cDNA based on production using RNA-dependent DNA polymerase isolated from avian myeloblastis virus. The first use of this for a plant virus cDNA was recorded by Kisselev et al., (1976) using cowpea mosaic virus. Soon after the transcription of RNA into DNA by avian sarcoma virus was described in detail (Taylor et al., 1976).

The technique consists in forming ^{32}PcDNA to a specific virus RNA and then incubating, under appropriate conditions, the test RNA's with the ^{32}PcDNA. The final mixture is then treated with nuclease S1 which is specific for single stranded nucleic acids. The remaining hybrids are then assayed by a suitable technique such as liquid scintillation spectrometry. Thus this method relies on assaying the hybrids formed rather than the extent of mismatching by competition as done with the use of RNA-RNA hybridisation.

Although a recent development, the technique has been used to examine the RNA's of different viruses (Demeure et al., 1977), strains of one virus (Gonda and Symons, 1978), the RNA's produced by infection with a single virus (Gould and Symons, 1977) and specific loci (Robbins et al., 1977). In all these examples of varying degrees of relatedness of RNA's, the use of this method yielded valuable information.

(c) Comparison of RNA and DNA molecular hybridisation. Gonda and Symons (1978) examined the two different techniques and they considered that there were 3 main disadvantages with the use of RNA compared to the use of DNA and these were as follows. Firstly when viruses with multipartite genomes were examined the dsRNA isolated was the total present and not fractionated into its components. The relative proportions of the dsRNA species are not necessarily the same as those of the ssRNA's. Secondly the use of dsRNA gives no idea of the amount of mismatching in the heterologous hybrids formed between the unlabelled competitor and the dsRNA, it only indicates the competitive displacement of the labelled RNA by the unlabelled heterologous competitor. Thirdly if a high background RNase resistance of the labelled RNA is obtained the detection of small amounts of sequence homology could be difficult. They consider that these factors could contribute to the fact that some strains of a single virus showed very few cases of partial homology and in some instances showed no homology using dsRNA. In experiments with cucumber mosaic virus using the dsRNA technique no homology was found between some strains and yet significant homology was found using cDNA.

1.5. Immuno-Electron Microscopy

With the advent of modern electron microscopy and negative staining techniques there was, naturally, an interest in viewing the reaction between antibody and antigen and in combining the techniques of serology and electron microscopy to obtain greater accuracy and sensitivity of results. This field became known as immuno electron microscopy (IEM) and the variations in methodology have been discussed by Milne and Luisoni (1977).

It is appropiate here to look at some of the major points in the development of the technique from 1968 to the present.

The original methods were developed primarily to study the antigen/antibody complex and consisted in reacting the antibody and antigen and putting the precipitate (often after a low speed centrifugation) onto the grid for EM examination. There were a number of disadvantages, not the least of which was that they were time consuming and that the low speed centrifugation precipitated impurities that degraded the image.

One method developed for examining infected plants for virus infection was to place a drop of negative stain on a grid and then pass a freshly cut leaf edge through the drop, dry and examine in the EM. Ball and Brakke (1968) essentially replaced the drop of negative stain by a drop of antiserum which had the advantages of requiring small amounts of antiserum (in the order of 5 µl) and the reaction between the virus and antibody could be directly observed.

The same authors later (Ball and Brakke, 1969) modified the technique to incorporate density gradient centrifugation. These "leaf dip serology" techniques were reviewed by Ball (1972) and this

methodology was used to determine strain relationships of some rod-shaped plant viruses (Langenberg, 1974).

An important advance was made when it was realised that the grid could be coated with specific antibody before a suspension of virus (or infected sap) was placed on the grid and that this would greatly increase the number of virus particles seen in the EM (Derrick, 1972, 1973a, 1973b).

Milne and Luisoni (1975) then found that once particles were on the grid and antibodies added they formed a "halo" (termed "decoration") around the virus particles that had reacted with the antibody. Then the techniques of grid coating and decoration were combined to give the best proof of a specific immune reaction in the electron microscope (Milne and Luisoni, 1977).

Since 1977 the method of IEM has been used successfully on a range of different viruses in different situations, such as the relative estimation of virus concentration in plant protoplasts (Beier and Shepherd, 1978); the detection of seedborne viruses (Hamilton and Nichols, 1978; Brlansky and Derrick, 1979); Reoviruses (Milne and Lesemann, 1978); Luteoviruses (Roberts and Harrison, 1979; Roberts et al., 1980); Tymoviruses (Lesemann et al., 1980); Nepoviruses (Roberts and Brown, 1980); Tombusviruses (Makkouk et al., 1981); human Rotaviruses (Nicolaieff et al., 1980) and various viruses that infect Rosaceae (Thomas, 1980; Kerlan et al., 1981).

During the early stages of the use and development of the method the times used to coat grids with antiserum and for virus acquisition were short, in the order of 10-15 minutes for each. Some improvement was made in this rapid method by the use of protein-A,

isolated from the cell walls of Staphylococcus aureus, to precoat the grid before the application of antiserum (Shukla and Gough, 1979). This modification increased the number of particles trapped on a grid compared with other rapid methods but was slightly less economical as far as antiserum is concerned.

When work was done with viruses that could not be detected by normal negative staining, because of their very low concentrations in sap, longer virus acquisition times became necessary and it was found that the time could be varied to suit the virus being studied (Roberts and Harrison, 1979). The longer acquisition times also made the method sensitive enough to detect various Nepoviruses in the nematode vectors in which there was no evidence of virus multiplication (Roberts and Brown, 1980).

Some attempts have been made to compare the sensitivity of IEM with that of other methods currently in use, the most sensitive of which was ELISA (Clark and Adams, 1977). Using a virus acquisition time of 60 minutes, Hamilton and Nichols (1978) found that in seedlots both IEM and ELISA could detect a 5% level of infection with pea seed-borne mosaic, but at infection levels below 5% IEM tests were positive and ELISA tests negative. Thomas (1980) looked at the detection of viruses infecting roses by using 4 serological tests: ELISA, latex flocculation assay, gel diffusion and IEM. The viruses all occurred in low concentration and could not be detected by negatively staining sap exudates. He used a virus acquisition time of 60 minutes and found the latex test up to 250x more sensitive than gel diffusion; IEM and ELISA up to 1,000x and 200x more sensitive than the latex test respectively.

In so far as detection of plant viruses in crude homogenates of infected tissue is concerned, IEM has proved to be of great value and sensitivity. Part of the reason for the great sensitivity is that in theory detection relies on the presence of a few particles only on the grid, whereas other methods rely on comparatively high numbers being present. Among the principal advantages that workers have found for IEM are that it is rapid, only small amounts of infected tissue are needed, crude extracts may be used, small amounts of antisera are used, it eliminates the interpretation of banding in a gel, and there is direct viewing of the reaction.

In some instances some workers have found that when viruses are present in low numbers on a grid it is an advantage to be able to scan the grid at very low magnifications. Often this is complicated by the fact that the contrast available is insufficient to see particles clearly, even after they have been decorated with specific antiserum. This has been overcome to some extent by a technique of double decoration in which the virus particles are first decorated with rabbit-anti-virus IgG and then treated again with sheep-anti-rabbit IgG (Kerlan et al., 1981).

This work has all been concerned with detection of virus infections as opposed to the identification of the virus particles that are trapped onto the grid. When an antiserum-coated grid is used to trap virus particles there is not only an increase in numbers of particles of the homologous virus, but particles of serologically related viruses are also trapped. Thus, when infected plant sap is used to trap viruses, it is not known whether the particles are those of the homologous or a related virus.

It would obviously be of great value to be able to identify the virus on the grid accurately, but there have been few detailed studies on this aspect since the development of IEM by Derrick (1973b).

The first of these attempts was made by Milne and Luisoni (1975) when they reported on two new procedures they adapted for plant virus work: decoration and clumping. In the first method particles of the virus are attached to the grid and then treated with antiserum for 15 minutes. If the virus was closely related to the antiserum the particles would be surrounded by a dark halo when stained and examined in the electron microscope. In the second method the antigen-antibody reaction proceeded in suspension and the sample was later collected for washing and staining. If the virus was related to the antiserum the virus particles would appear in clumps on the grid but would be spread more evenly if not related.

Later (Milne and Lesemann, 1978) a comparison was made of these two methods and the technique of counting particles trapped on a grid. They found the numbers of particles trapped varied considerably from one experiment to the next and thought that the variations were more probably due to factors not well controlled or understood rather than to differences in serological relationships. The method did, however, differentiate between the two groups of viruses they used, but did not distinguish between the members of each group. Clumping and decoration also made the same distinction.

The points made by Milne and Lesemann (1978) about the 3 methods are that virus particle counting can be very time-consuming and subject to quite an amount of variation for reasons not yet established. Decoration was the most reliable technique as, in principle, only one particle had to be seen to get a result. Clumping

was occasionally of limited value, especially where only small numbers of particles were available.

During the work they attempted to compare the results from IEM with those from gel diffusion and slide precipitin tests and found that with the latter two methods they could not get a reaction unless the virus present in 50 g of starting material was concentrated to 0.1 ml. With IEM they could use less than 1 g of tissue and no concentration was necessary.

Many aspects of the trapping of Tymovirus particles were investigated (Lesemann et al., 1980) using purified preparations and preparations containing plant sap. They concluded that there was a linear relationship between log virus concentration and log number of particles trapped; that counting should allow more accurate comparisons of concentrations of virus particles than conventional absorption methods; that the heterologous reactions were only detected with those viruses that showed a very close relationship in gel diffusion tests; unknown factors led in some instances to aberrant results and had to be discarded.

Roberts et al. (1980) attempted to use IEM to determine the relationship of an isolate of potato leaf-roll virus by comparing it with 11 antisera to different Luteoviruses. To do this 2 methods were used: (1) a comparison of the number of virus particles trapped in a standard area (Roberts, 1980) and (2) a combination of the clumping and decoration methods of Milne and Luisoni (1975). This involved incubation of the virus with dilutions of the antisera prior to putting the preparation on the grid and then determining the greatest antiserum dilution that coated all particles with an anti-body.

As with Milne and Lesemann (1978) the particle counts of Roberts et al. did not give a very accurate separation of the antisera but they could put the Luteovirus antisera into 4 groups based on the particles present. The categories of particle numbers were large (>8,000 per standard area), moderate (4,000-6,000), smaller (400-700) and few (80-150). A similar grouping pattern was given by the "clumping/decoration" technique. Considering that, in reality, all that was being examined was the antibody coating on the virus particles (i.e. the decoration) it would have been more economical of materials and time to have decorated particles on a grid.

Concluding Remarks

Over the years there have been many techniques developed for the detection and identification of plant viruses, by analysis of various properties of the particles. While they all have a part to play in the development of knowledge of viruses, their biology and classification, a lot of the methods are highly specialised and some are complicated and of limited practical value in the general area of virus detection and diagnosis.

There have been two major developments that have been of major significance in the history of virology. The first is the use of electron microscopy in general and negative staining (Brenner and Horne, 1959) in particular. The second is the use of serology that has enabled relationships between viruses to be determined and has been used to improve detection and diagnosis of human, animal and plant viruses.

The combination of these two methods is a great advantage to the science of viology and can combine the specificity of the serological reactions with the sensitivity of the electron microscope

in that the reactions can be observed at the level of a single virus particle. A lot of the confusion resulting from non-specific serological precipitates is removed as the microscope enables the observation to be made as to whether virus particles are involved or not. The combination of the two offers many advantages in the rapidity of the tests, the economy of reactants and detection and identification of viruses in concentrations that were previously too low to be detectable.

The general method of trapping particles onto an antiserum coated grid (Derrick, 1973b) was originally called "Serologically specific electron microscopy" (SSEM). Since then the method and variations such as clumping and decoration have been known by the above name and a variety of others such as "immuno-electron microscopy" (IEM), "immune electron microscopy", "immuno-sorbent electron microscopy" (ISEM), and still other names that have not proved as popular as those above. Some of the terminology is geographically determined as SSEM is mostly used in the U.S.A. and ISEM seems somewhat more popular in Great Britain.

As the original description referred to the method as SSEM this has some claim to precedence but it only referred to trapping particles onto a grid. Since then many variations and modifications have widened the scope of the method considerably and some of them (e.g. clumping) are not really "immuno-sorbent" but do involve immunological methods. For these reasons I will refer to the technique in general, covering all the variations and modifications, as immuno-electron microscopy (IEM).

The method in its use to date has proved extremely valuable in detecting viruses in infected tissues more efficiently than has

any other technique. It has also been able to separate viruses into related groups but has not been very successful in separating closely related viruses or detecting very distant relationships.

There has, for some reason, been very little work done to compare the various methods now in use in different laboratories and to examine the advantages and deficiencies of each and the reasons for them. There has also been very limited use of what should be a very valuable and rapid tool for undertaking surveys of viruses in a field situation.

In a diagnostic sense counting particles seems to be the least useful method of application and is subject to variations for reasons many of which have not been elucidated, though one obvious cause of variation is the concentration of particles in the host.

Likewise the clumping technique seems to be subject to adverse results due to the concentration of virus in the sample. Decoration holds the most promise for diagnostic use as only a few virus particles are necessary to obtain a result.

With the above background, this study set out to examine IEM in its many aspects and to attempt to improve the diagnostic use of the method generally. It was designed to compare the variations in the method, to look at its use in increasing the numbers of particles for population size studies, to examine the diagnostic possibilities and to see how the method could be best used in a field survey situation in which large sample numbers and greatly varying virus concentrations are involved.

The Tobamovirus group was used for most of this work because a range of isolates was available, infected tissue contains a high

concentration of virus, the members of the group are relatively stable and can be manipulated easily in so far as host inoculations, purification etc. are involved, and there is a large amount of information available about the viruses with which to compare the results.

2. MATERIALS AND METHODS

"The joy of research must be found in doing, since every other harvest is uncertain."

Theobold Smith.

2. MATERIALS AND METHODS

The viruses used, their known relationships to each other, purification and antiserum production are all summarised by Pares and Whitecross (in press).

2.1. Viruses

The four viruses used principally in these studies were the U1 and U2 strains of tobacco mosaic virus, the type-strain of tomato mosaic virus and a local isolate of Odontoglossum ringspot virus. The first 3 viruses were obtained as frozen leaf tissue from Dr A.J. Gibbs, Research School of Biological Sciences, Australian National University, Canberra. The isolate of Odontoglossum ringspot virus was obtained from an orchid plant that was reportedly a species of Laeliocattleya growing in a glasshouse at the Commonwealth Nursery, Yarralumla, A.C.T., and was isolated from local lesions produced by mechanical inoculation onto Chenopodium quinoa.

2.1.1. *Maintenance of isolates.*

All viruses were maintained in plants growing under glasshouse conditions. The two isolates of tobacco mosaic virus were maintained in Nicotiana tabacum, the U1 strain on cv. White Burley and the U2 strain on cv. Samsun. The isolate of tomato mosaic virus was maintained in Lycopersicon esculentum cv. Grosse Lisse, and the orchid virus in Chenopodium quinoa.

The isolates were also kept as leaf tissue and a leaf macerate in phosphate buffer stored at -20°C.

For transfer of the viruses to healthy seedlings, infected leaf tissue was macerated with a mortar and pestle in 0.06 M phosphate buffer (PB) at pH 7.0, ca. 3 ml per g tissue. A small amount of celite

was added to act as an abrasive. The macerate was rubbed onto host leaves with a finger and the leaf surface washed immediately with tap water.

2.1.2. *Terminology for the viruses.*

Throughout the remainder of this thesis the viruses will be referred to by the following abbreviations:

U1-TMV - the U1 (type) strain of tobacco mosaic virus.
U2-TMV - the U2 strain of tobacco mosaic virus.
ToMV - Tomato mosaic virus.
ORSV - Odontoglossum ringspot virus.

2.1.3. *The serological relationships between the viruses.*

Based on Gibbs (1977) and Paul (1975) it would appear that U2-TMV and ToMV are closely related to each other and each of these is more distantly related to U1-TMV than they are to each other. ORSV is quite distantly related to all three of the other viruses.

2.1.4. *Virus purification.*

The following schedule, using the principle of differential centrifugation, was found to be satisfactory for all 4 viruses.

1. Infected leaf tissue was macerated in a blendor with PB containing 0.1% thyoglycollic acid (TGA). The PB was used at the rate of ca. 4 ml/g tissue. The resulting macerate was then expressed through 4 layers of gauze.

2. To the above extract was added n-butanol to give a final concentration of 8.3%. This was added drop-wise to the extract while stirring and then left stirring for an additional 60 minutes at room temperature (R.T.).

3. The resulting preparation was centrifuged at 4,000 rpm for 30 minutes, the supernatant removed and then centrifuged at 27,000 rpm for 90 minutes in a Beckman Ti60 rotor. The supernatant

was discarded and the pellets resuspended overnight at 4°C in 0.03 M PB, using 1 ml of buffer per pellet.

4. The cycle of low speed/high speed centrifugation was repeated.

5. The resulting resuspended pellets were then layered onto the surface of a 10-40% sucrose density gradient tube, 1.5 ml of virus suspension per tube. The gradient tubes were then centrifuged at 27,000 rpm for 150 minutes in a Beckman SW27 rotor.

6. The bands in the gradient tubes were removed by piercing the side of the tube and extracting them with a hypodermic syringe. The suspension was then centrifuged at 27,000 rpm for 90 minutes to remove any sucrose and the pellet resuspended as previously described.

2.2. Antisera

2.2.1. *Preparation and Storage.*

Antisera to all viruses were prepared in the same way, in rabbits. The inoculation schedule was: an intravenous injection of 0.2 ml of the above virus suspension, followed 16 days later with an intramuscular injection of a mixture of 0.25 ml virus, 0.5 ml Freund's complete adjuvant and 0.2 ml distilled water. A second intramuscular injection made up in the same way was given 15 days later and a final intravenous injection of 0.2 ml virus was given after a further 2 months. The rabbits were then bled 14 days later.

The tubes of blood were left at room temperature to clot, the serum poured off and centrifuged at low speed, the supernatant diluted 1:1 with glycerol and stored at 4°C.

2.2.2. *Terminology to be used for antisera.*

In the remainder of the text the antiserum to a particular virus will be referred to by the prefix "a." before the abbreviated name of the virus to which it has been prepared. As an example the antiserum to the virus ToMV will be a.ToMV.

2.3. Grid Preparation and Electron Microscopy

The basic aspects of the method will be described here and variations and specific details will be described in the appropriate section. Any buffers and other solutions used are given in 2.5.

For all electron microscopy 300 or 400 mesh copper grids were used and before use were coated with formvar and carbon.

2.3.1. *Normal negative staining (leaf crush).*

A small piece (ca. 3 mm x 3 mm) of infected leaf tissue was crushed in one or two drops of 2% ammonium molybdate (AM) on a glass light-microscope slide. A grid was then placed, film side down, onto the drop of macerate for a few seconds, removed and dried by touching the edge to a piece of filter paper.

In some instances, instead of using the "leaf crush" method, preparations were made from the frozen leaf macerate. To do this equal amounts of the thawed preparation and the 2% AM were mixed on a glass microscope slide. This was then put onto the grid in the same manner described for the leaf crush. When frozen purified preparations of the viruses were used they were treated in the same way.

There was no detectable difference between the 2 types of preparation described above for the unpurified preparations. Because of this, and in order to distinguish these negatively stained preparations from those prepared by IEM methods, they will be referred to as "untrapped".

2.3.2. *Immuno-electron microscopy.*

The method can be divided into 5 separate stages: (1) coating the filmed-grid with antiserum; (2) trapping the virus onto the coated grid (virus acquisition); (3) treatment of the particles with antiserum (decoration); (4) treatment of the particles with labelled antibodies (GLAD); (5) negatively staining the final preparation.

Reactants were applied to the grid surface by placing 5-20 µl drops of the reactants in a plastic petri-dish, and floating the grids (film side down) on the surface of the drops. After the appropriate time the grids were removed, washed with 20 drops of 0.03 M phosphate buffer (pH 7.0), and drained (but not dried) by touching the edge briefly to a piece of filter paper, before being placed onto a drop of the next reactant.

Antiserum coating: For most routine experimental work the antisera were diluted in carbonate buffer (CB). The grid treatment was always done at room temperature, the treatment time depending on the particular experiment.

Virus acquisition: The virus samples were usually diluted in 0.06 M phosphate buffer (PB), when necessary. The crude sap preparations were made by macerating leaf tissue in this buffer at the rate of 3 ml per g tissue, and this is the starting point for any dilutions mentioned in the text for virus preparations.

The virus acquisition time (VAT) varied greatly from 10 minutes to 18 hours depending on the experiment. The shorter times (up to ca. 4 hours) were done at room temperature but the longer times had to be done at 5°C to avoid evaporation of the droplets.

Decoration: This step used antiserum diluted in CB, and the treatment time was short, in the region of 10-20 minutes and was done at room temperature.

GLAD: This aspect is in the developmental stages and is more fully described in 2.4 and section 6. It was always done at room temperature.

Negative staining: After the grids had been prepared, and whether decorated or not, they were washed in 20 drops of PB, and then, without draining, they were treated with 6 drops of 2% AM, drained by touching the edge to a piece of filter paper and allowed to dry before examination in the electron microscope.

2.3.3. *Electron microscopy.*

All the electron microscopy was done using a Philips EM301 electron microscope, operated at 60 kV. Where necessary electron micrographs were taken on 35 mm film, and for the measurements in section 4 the negatives were enlarged 7x to produce prints at a magnification of 92,000x. Measurements were done using a mm rule in the early stages, but then later most were done using an image analyser.

Counting of either virus or gold particles was originally done both by direct viewing on the fluorescent screen via the binocular of the electron microscope, and by examining electron micrographs. The same results were produced by both methods, so that subsequently counts were done only at the electron microscope.

In order to obtain ready comparisons from one experiment to another, in which the density of the particles on the grid may have been different, it was necessary to utilise a figure that was independent of this variation. To do this the counts obtained from the

homologous virus-antiserum combination were divided by the counts obtained from the heterologous combination. As this ratio is designed to illustrate relationships that may exist, I will refer to it as the "affinity ratio".

2.4. The GLAD Technique

This technique is one developed during the research described in sections 3-7, and named and described by Pares and Whitecross (in press). The method will be described here in greater detail than in that publication.

2.4.1. *Preparation of the colloidal gold/protein-A (G/pA)*

Much of the developmental work was done using material kindly supplied by Mr S. Craig and Dr A. Millerd, Division of Plant Industry, CSIRO, Canberra. This and subsequent preparations were made by the following method:

Where possible the flasks, etc. used for the gold solutions should be of siliconised glass as the gold can adsorb to a glass surface.

Solutions needed:

50 ml	0.01%	$HAuCl_4$ (gold tetrachloroauric acid)
50 ml	1.0 %	Sodium citrate
100 ml	0.2 M	Potassium carbonate
100 ml	10.0%	Sodium chloride
50 ml	1.0%	Polyethylene glycol (Carbowax 20 M)

Phosphate buffered saline, pH 7.0 (PBS).

Method: Bring the 50 ml of $HAuCl_4$ solution to the boil and add dropwise by pasteur pipette a mixture of 0.6 ml of the sodium citrate and 1.4 ml of distilled water, and leave to boil for 5 minutes. The solution should change from clear to burgundy colour. The flask is then cooled under running water and adjusted to pH 6.9 with 0.2 M K_2CO_3,

using pH papers as gold will damage the electrodes of pH meters. This should produce colloidal gold with a diameter of about 20 nm.

The colloidal gold has then to be stabilised with protein-A by putting 0.5 ml of the gold colloid into each of 8 tubes, and to each is added 0.1 ml of one dilution of a solution of protein-A in distilled water. The dilution series of protein-A is a two-fold one from 1.0 mg/ml - 0.007 mg/ml. The tubes containing gold and protein-A are left 5 minutes and then 0.5 ml 10% NaCl is added to each. The solution should remain a burgundy colour but if there is insufficient protein-A it will go a blue colour. The highest dilution of protein-A that maintains the burgundy colour is selected and protein-A is added at that rate + 20% to an aliquot (e.g. 10 ml) of the colloidal gold. After leaving 2-3 minutes, 0.2 ml of 1% polyethylene glycol is added per 10 ml of stabilised gold.

This preparation is then centrifuged at ca. 40,000 rpm (for a Beckman 40.3 rotor) for 40 minutes, the pellet resuspended in ca. 3 ml PBS + azide + polyethylene glycol (0.2 mg/ml). This is then centrifuged at ca. 8,000 rpm for 3-4 minutes.

2.4.2. *Preparation and use of the colloidal gold - protein A - antiserum conjugate.*

The G/pA prepared as in 2.4.1. was used either as prepared, or diluted when required in PB. It was mixed in a small tube with antiserum, usually at the rate of 1 vol G/pA and 2 vol antiserum, and then left to stand at room temperature before use. This conjugate was prepared each day it was used and no experimentation has been done on the shelf-life of the conjugate. The conjugate was then used by putting a 5 µl drop in a plastic petri-dish and floating the grid (with virus) on it for ca. 30 minutes. The grid was then removed, washed in 20 drops PB followed by 6 drops 2% AM.

For simplicity the conjugates will be referred to by prefixing the antiserum by "G/", as an example gold/protein-A conjugated to the antiserum to tomato mosaic virus would be "G/a.ToMV".

2.5. Buffers and Solutions

There are a few solutions that were used frequently in the experiments and not described above, and these are:

2.5.1. *Phosphate Buffer (PB).*

2.84 g	Na_2HPO_4
6.35 g	KH_2PO_4
1 l	distilled water

pH 7.0.

When used for washing grids this buffer was diluted 1:1 with distilled water.

2.5.2. *Carbonate Buffer (CB).*

1.59 g	Na_2CO_3
2.93 g	$NaHCO_3$
1 l	distilled water

pH 9.6.

2.5.3. *Ammonium Molybdate (AM).*

2 g $(NH_4)_6\ MO_7O_{24},\ 4H_2O$

100 ml distilled water

The AM was thoroughly dissolved in the water by stirring for ca. 30 minutes. There is always a residue that remains undissolved and this was removed by filtering through filter paper and storing in a stoppered bottle at RT. If the solution became dirty it was then re-filtered. The pH was not adjusted.

2.5.4. *Saline Sodium citrate (SSC).*

This is a preparation made from a combination of 0.15 M sodium chloride and 0.015 M sodium citrate in distilled water.

3. COMPARISON OF METHODS

"Fixity of purpose calls for flexibility of method."

Anon.

3.1. Introduction

The introductory literature review (section 1) outlined a number of minor and major developments in the basic technique of IEM since it was first developed by Derrick (1973b). The two major changes have been the improvement of the short method by precoating grids with protein-A (pA) (Shukla and Gough, 1979), and increasing sensitivity by using dilute antisera and long coating and virus acquisition times (Roberts and Harrison, 1979).

The range of modifications used in various laboratories can be divided into 2 groups, (1) the rapid methods that are completed in ca. 1 hour or less and (2) the slow methods that take from 3-18 hours to complete.

The rapid methods were compared during the development of the use of pA by Shukla and Gough (1979), but there has been no published comparison of the slow methods either as a group or as compared to the rapid methods.

When anyone is intending to utilise IEM for a virus research or identification project the question that should, and does, arise is "What is the best method to use?" To this question there is, at present, no unequivocal answer and it becomes a matter of selecting one of the published methods and following it without really knowing whether it is the best to use in that particular situation.

In some instances it may be desirable to modify a technique for a particular purpose but there is no information on the interactions of such factors as the dilution of antisera, use of pA and variations in coating and virus acquisition times. This then makes modification more difficult than it should be.

There have been some statements made that the time regimes may not be optimal (Shukla and Gough, 1979; Milne and Luisoni, 1977) and on the possible effects of using pA (Shukla and Gough, 1979) but there was at no time any real effort to see whether the statements fitted the known facts, nor to attempt proof by further experimental work.

The experiments reported in this section were done to see whether there were any major differences between 7 methods reported and to determine what factors might be involved in these differences.

3.2. Comparison of IEM Methods

When a comparative analysis is being made of the efficiency of the methods in detecting virus particles, one of the first steps is to take a range of the available methods and to test them at the same time against the same virus preparation. In order to do this 3 rapid methods (i.e. methods that take less than 1 hour) and 3 slow methods (that require 2-18 hours VAT) were chosen. In addition a modification to one of the slow methods was also tested. The modification was a reduction in the VAT of Plumb's (1980, pers. comm.) technique from overnight to 120 minutes.

3.2.1. *Materials and Methods.*

For ease of reference the methods will be referred to by the initial letters of the originator and are shown in Table 3/1.

Table 3/1. Methods used in initial comparison of IEM techniques.

METHOD		REFERENCE
D		Derrick (1973b)
M-L	Rapid	Milne and Luisoni (1977)
S-G		Shukla and Gough (1979
R-H		Roberts and Harrison (1979)
P	Slow	Plumb (1980 - pers. comm.)
T		Thomas (1980)
P(S)		Modification of Plumb's method.

The methods were all carried out as described in the references except that in all instances the leaf tissue was ground in the appropriate buffer at the rate of 50 ml per 1 g leaf tissue. This was to ensure a uniformity of virus concentration for all methods and was chosen so that the virus preparation was dilute enough for there not to be extremely large numbers of particles over the grid. The virus used was U2-TMV.

3.2.2. *Results.*

Because the aim of the experiment was to determine any major differences rather than minor but statistically significant differences, no counts were made on particle numbers per unit area of the grid. The grids were assessed visually and this enabled some obvious differences to be noted and some generalisations to be made.

Firstly the slower methods seemed to trap more particles than the rapid methods. Of the slower methods, P seemed to be one of the best whether the VAT was overnight at 4°C or 120 minutes at room temperature. Of the rapid methods S-G was obviously vastly superior to the other 2. There were, in fact, so few particles present on the grids of methods D and M-L that the experiments were repeated to check that no mistake had been made, however the results verified those previously obtained.

In spite of many efforts in this and other experimental work to utilise Derrick's method, the preparations always had a lot of contamination present making electron microscopy very difficult.

As well as being by far the best rapid technique, method S-G appeared to be almost as good as method T, which took 5 hours.

3.2.3. *Discussion.*

Even though the virus concentration in sap was diluted 1:50, there was still a reasonable concentration of particles present in the preparation used. At this level of virus concentration all of the methods gave a greater density of particles on the grid than untrapped preparations of crude sap. This confirms the original claim for each of the methods.

There is little doubt that for the most efficient detection of particles, at the concentrations used here, and using a rapid method, the S-G method is the best. The only disadvantages that seem to be associated with this method are firstly having an extra step in the process, for which a supply of pA is needed, and secondly using much more antiserum. It is used at a dilution of 1:20, whereas most of the slower methods use antiserum at dilutions in the region of 1:1,000.

3.3. An Examination of some of the factors involved in the relative sensitivities of the Rapid and Slow Methods

One of the real values of trapping viruses onto antiserum-coated grids is its ability to detect viruses that occur in concentrations too low to be detected by other means. Having examined the range of methods that have been developed, it is now of value to examine the better ones under conditions in which IEM has the greatest value. Once this is done some of the factors involved in making one method better than another will be examined.

In this series of experiments the virus preparations were much more dilute than previously used. Counts were done in the electron microscope, 10 random binocular fields were counted and the mean of the 10 such counts used for comparisons.

3.3.1. *Experimental.*

a) The first step in this series was to examine the best of the rapid methods (Shukla and Gough, 1979), the best of the slow methods (Plumb, pers. comm.) with a 120 minute virus acquisition, and the method of Plumb with the usual overnight virus acquisition. These will be referred to as rapid, medium and slow, respectively.

Because the method of Plumb has not been published to date and because it proved to be one of the better methods, I will now summarise the main details:

Grids are coated for ca. 90 minutes with antiserum diluted 1:1,000 in carbonate buffer; the grids are then washed in 0.06 M PB and put onto drops of the virus preparation overnight at 4°C. The virus preparation is made by grinding infected leaf tissue in 0.06 M PB. After virus acquisition the grids are washed in 0.03 M PB and then negatively stained.

The three methods mentioned above were used to trap particles from a preparation of U2-TMV at dilutions of 10^{-6} and 10^{-10}.

Grids prepared using the virus at 10^{-6} were compared qualititatively and there were more particles present using the slow method, a reasonable number with the medium one and obviously far fewer with the rapid technique.

Counts were made on the grids from the 10^{-10} virus preparation and the numbers (± standard error of the mean) were, slow:25.3 (±1.9); medium:14.4 (±0.75); rapid:2.3 (±0.4).

This establishes the greater sensitivity of the slower method but raises the questions as to whether the difference is due to the differences in virus acquisition time, the antiserum coating time or

the use of protein-A (0.1 mg/ml).

b) The second point to be examined was the improvement gained when the virus acquisition time is increased for the rapid method. Using virus preparations and concentrations greater than used above Shukla and Gough (1979) make the comment that the acquisition time they used may not be optimal but did not get much of an increase in particle numbers with increasing VAT.

To look at this point the Shukla and Gough (1979) method was used with U2-TMV at dilutions of 10^{-8}, 10^{-9} and 10^{-10} and acquisition times of 10 minutes and overnight.

These virus dilutions proved to be almost at the limits of sensitivity for the method being tested and there were virtually no particles present on the grids using a 10 minute trapping time. There were also very low numbers present with overnight trapping but they were far easier to detect than with the shorter trapping. Even though there were very few particles present it does demonstrate that under limiting conditions the longer VAT does give a considerable increase in sensitivity to the rapid technique.

c) Even though the long VAT greatly improves the rapid method, is it as sensitive as the slow method? To look at this question the rapid method, the rapid method with an overnight VAT and the slow method were used to trap viruses from a 10^{-6} preparation of U2-TMV. Sufficient particles were present to enable counting and the means for the treatments were (± standard error of the mean), slow method 10.7 (±0.54); rapid method (long VAT) 4.7 (±0.72); rapid method 0.1 (±0.1), indicating that either the long coating time (ca. 90 minutes) or the absence of protein-A increase the efficiency of the standard slow method.

d) The 2 factors of (i) presence or absence of protein-A and (ii) the coating time, were investigated using the virus ToMV at a dilution of 10^{-4} and its antiserum at 1:20 and 1:1,000 at coating times of 10 and 90 minutes. Each of the combinations of antiserum dilution and coating time were used on grids treated and untreated with protein-A. The results of the particle counts for each of the combinations are shown in Table 3/2.

Table 3/2. Counts of virus particles in a comparison of the use of pA using 2 antiserum coating times. The figures given are the means of 10 counts ± standard error of the mean.

COATING TIME	ANTISERUM DILUTION	
	1:20	1:1,000
10 min. (+pA)	33.6 ± 2.5	21.5 ± 0.7
10 min. (-pA)	11.2 ± 0.9	31.5 ± 2.0
90 min. (+pA)	33.4 ± 1.7	22.0 ± 1.1
90 min. (-pA)	13.6 ± 1.1	52.2 ± 4.0

3.3.2. *Discussion.*

Due to the differences in virus concentration that may occur in the samples used for each of these experiments, the counts cannot be compared between each experiment even though the dilution used may have been the same.

It is clear that the shorter trapping times are not as efficient as the overnight treatment when the virus concentration is very low, conditions under which IEM has its greatest potential for use in virus detection. It appears from this that one of the important ways to increase sensitivity is to employ a long virus acquisition time.

Counts made in the first part of this series of experiments (3.3.1. a) confirmed this trend; a 120 minute trapping time only

picked up ca. 57% of those trapped by the overnight VAT. The rapid method only trapped 9% of the particles trapped by the long method and 16% of those trapped with a 120 minute VAT.

The second part of the experiment shows that part of the reason for the superiority of the longer method is due to the longer VAT used and that a longer VAT can greatly improve the rapid method of Shukla and Gough (1979) under conditions of very low virus concentrations such that the standard rapid method fails to detect the presence of any virus particles.

There is, however, more involved in the greater sensitivity of the long method than simply the longer trapping times used as the third part of the experiment illustrates that with an overnight VAT the method of Shukla and Gough (1979) trapped only 44% of the number of particles trapped by the longer method.

This could mean that there were more attachment sites on grids that were coated with antiserum without using protein-A, and for a longer time. The fourth part of this experiment was designed to examine this.

The results show up a few important points about the IEM technique and the factors involved in maximising the sensitivity.

Firstly for a given coating time the use of antiserum diluted 1:1,000 and used without pA is as good, or better, than using antiserum at 1:20 with pA. From this one set of experiments it would appear that the most sensitive method is to coat the grids with antiserum at 1:1,000, without protein-A and use a coating time of 90 minutes.

It seems that, at least when pA is used to coat the grid before antiserum, the antibody coating must take place very quickly, within the first 10 minutes. In fact if a long VAT is used, then using pA and coating with antiserum for 10 minutes is no more efficient (and uses more antiserum) than using antiserum at 1:1,000 for 10 minutes on a grid that has not been pre-treated with pA.

Thus it would appear that the VAT is not the only important factor in giving the longer methods an advantage over the method of Shukla and Gough (1979) and the method of coating the grid with antiserum is also important in maximising the detectability of virus particles.

3.4. General Discussion

The process of trapping virus particles from sap with antiserum-coated grids is basically that of inspissation and the only way to judge the results of changing any facets of the method is by comparing the density of the virus particles either by visual assessment and comparison or by counting the number of virus particles in a given area of the grid.

Because the coating of either pA or IgG molecules cannot be directly visualised, any statements about them have to be made by inference from data on comparative particle densities.

Before commenting on the comparisons made between the various methods it should be noted that all methods tested proved to be superior to the use of untrapped grids.*

When the method of pre-coating grids with pA was developed by Shukla and Gough (1979) the only published techniques with which they could be compared were rapid ones that used the coating anti-

* see Addendum, Plate 1.

serum at dilutions of 1:10 (Derrick, 1973b) or 1:10-1:100 (Milne and Luisoni, 1977). My work has shown that the rapid methods as described originally are fairly ineffectual compared to the technique of using protein-A, but if the antiserum is used at 1:1,000 the rapid methods without pA are greatly improved (particle numbers trapped increase by ca. 3x) and are as effective as the method of Shukla and Gough. Although no work has been published on this, the literature indicates that there is a current awareness of the fact that antiserum should be used at 1:1,000 and most recent work using the Derrick method now utilises this dilution.

Thus, at the time, the claim of Shukla and Gough was correct that pA greatly improved the sensitivity of the method, but in the light of my results and current usage the method does not really offer any advantage. More improvement can be made by increasing the virus acquisition times.

The choice then is not simply a matter of whether it is thought desirable to use a rapid or slow method, as this series of experiments has shown that virus concentration in the sap is a very important consideration. If the viruses are present in reasonable concentrations and economy of time is an important factor then a rapid method using antiserum at 1:1,000 would be satisfactory.

The Potyvirus group are generally regarded as occurring in relatively low concentrations in plant sap. One member of this group is sugarcane mosaic virus and the fact that Shukla and Gough (1979) could satisfactorily detect this virus formed the basis for their statement that their method was "especially useful for viruses that are present in plants in low numbers". It should be noted that the Potyviruses can be detected in infected plant sap by the untrapped methods of normal negative staining.

The concept of "low numbers" is a relative one. In many instances viruses are present at such low concentrations in plant sap that they cannot be detected by normal negative staining and can only be detected after elaborate and time-consuming purification procedures. The low concentrations can be an intrinsic property of the virus (e.g. Luteoviruses such as potato leaf roll) or can occur with any virus at certain stages during the infection process. It is under such limiting, but by no means uncommon, conditions that IEM is invaluable and a rapid method may not be sufficiently sensitive.

Both Milne and Luisoni (1977) and Shukla and Gough (1979) admit that their VAT's of 10-15 minutes may be too short to give maximal combination of virus and antibody. It would thus appear that for very low concentrations of virus there are two possibilities for improving detection and these are (a) increasing the VAT or (b) using one of the slower methods that have longer coating times and longer VAT's.

This series of experiments demonstrated that simply increasing the VAT in the method of Shukla and Gough (1979), while increasing the particle density on the grid, was not as sensitive as the longer method. This deficiency could not be overcome by increasing the coating time with antiserum, a modification which in fact had no effect on particle density.

These facts would strongly indicate that the number of sites for virus attachment using pA is less than that in a slow method and that it cannot be increased by applying the antiserum for longer periods of time. It would thus seem essential that, under limiting conditions of virus concentration, a long method with at least an overnight (ca. 18 hour) VAT must be used to maximise the chances of success.

It is, therefore, not surprising that the longer methods were developed and used in laboratories that were working with viruses that were present in very low concentrations such as potato leaf roll and other Luteoviruses (Roberts and Harrison, 1979), Prunus necrotic ringspot (Thomas, 1980), viruses in nematode vectors (Roberts and Brown, 1980) and cryptic viruses (Plumb, pers. comm.).

My results have highlighted a number of points that require further discussion and these points are: (1) On grids not treated with pA an antiserum dilution of 1:1,000 gives a greater particle density than more concentrated antisera (e.g. 1:20). (2) When pA is used on a grid the antiserum coating time does not affect particle density and maximum antiserum covering seems to occur quickly, in about 10 minutes or less; and (3) Using the appropriate antiserum dilutions the number of potential virus attachment sites appears to be less when pA is used than when it is absent from the grid.

In the following discussion of these points an antiserum concentration of 1:20 will be referred to as "concentrated" and 1:1,000 will be referred to as "dilute".

The fact that, in the absence of pA, dilute antiserum was more effective than concentrated antiserum, has been frequently observed with a number of different viruses. Examples are Barley yellow dwarf (Paliwal, 1977), Tymoviruses (Lesemann et al., 1980) and oat sterile dwarf virus (Milne and Lesemann, 1978). The basic reason for this phenomenon has not been determined but Lesemann et al. (1980) consider that the inhibition effect was not related to serum titres and Milne and Lesemann (1978) assumed that at higher concentrations serum proteins were present in sufficient quantities to compete for sites on the grid.

The surface of an untreated grid that is used for IEM consists of a uniform layer of carbon. Thus IgG molecules would be able to attach to any part of the carbon film by a non-specific rather than by a specific coupling mechanism. It would therefore be expected that if any inhibitory agent were present in concentrated antiserum it would be most likely to act by directly competing for space on the grid surface. The serum proteins implicated by Milne and Lesemann (1978) would act in this manner. Antiserum dilution would proportionally reduce the effects of any such competitive inhibitor.

Under a system such as that proposed above, in which IgG attachment to the grid was non-specific, there would be extremely large numbers of potential attachment sites on the grid surface. Attachment would depend on chance contact between an IgG molecule in suspension and the surface of the grid. At first such random contact would be expected to occur frequently but with time the contact frequency would decrease but nevertheless still occur. Under such conditions there would continue to be more IgG molecules attached to the grid as the coating time is increased from 10 minutes to 90 minutes.

My results show that this is in fact what happens, and so are not inconsistent with the above proposal of random, non-specific attachment. It would further be expected that if there were no competitive inhibitor present in the antiserum then the same process would happen with concentrated antisera but the initial phases would happen more quickly due to the greater numbers of IgG molecules present in the suspension.

Before taking this point any further it is necessary to look at some of the known properties of pA and then to compare the expected and actual results of its use in IEM. The pA attaches

specifically to the Fc end of IgG molecules (Forsgren and Sjöquist, 1966, 1967). The molecule of pA is very elongated and, in theory, is able to react with a maximum of 4 Fc portions (Roth et al., 1978). In practice, however, it seems that it reacts with only 2 Fc portions of IgG molecules (Sjöquist et al., 1972).

When a grid was coated with pA it would be expected that, (1) because there are specific attachment sites for IgG molecules, the effect of any competitive inhibitor would be nullified; (2) with short antiserum application times, the concentrated antisera would be more effective than the dilute, because of the increased chances of contact between an IgG molecule and an attachment site; (3) with long application times there would be a slowly diminishing difference between dilute and concentrated antiserum and that given time eventually there would be the same number of IgG molecules on the grid with both concentrations of antisera; (4) with long coating times, the comparison of particle density both with and without pA would indicate the relative "holding capacity" of the grids in regard to numbers of IgG molecules.

The improvement in efficiency of concentrated antiserum when pA is used to coat the grid first would indicate that the effect of any inhibitor is nullified and that the inhibitor was acting as a competitor for space under conditions of non-specific attachment to the grid. When pA was used the attachment of IgG occurred very quickly with both concentrated and dilute antisera and there did not appear to be any improvement with time, which is not what was expected. This could mean that there was a strong attraction force between the IgG molecule and the site on the pA, and that there was therefore no improvement with time because the spatial restrictions do not apply with a strong attracting force. It could also mean that there were

fewer attachment sites on a pA-coated grid and therefore a greater spacing between them, a feature that would also reduce any spatial restrictions. The reason may be a combination of these two factors, a strong attraction force and fewer attachment sites.

There was no apparent increase in attachment of IgG with time when pA was used and there was no gradual diminution of the difference between the two dilutions of antisera. The difference between the two dilutions (ca. 65%) must be a reflection of the differing concentrations that were applied.

Though the use of pA greatly improved the effectiveness of concentrated antiserum it actually decreased the effectiveness of the dilute antiserum. Thus in a situation where the effect of any competitive inhibitor was minimised (i.e. by using dilute antiserum), there appeared to be less IgG attached to pA-coated grids than on grids not pretreated with pA. This is another piece of evidence that would indicate there are less potential attachment points on pA-coated grids than on uncoated grids.

Based on the fact that the pA:IgG combining ratio is 1:2, Shukla and Gough (1979) considered that there would be more sites for IgG on a pA-coated grid than when grids were treated with antiserum alone. I do not agree that the combining ratio warrants those conclusions and in fact the opposite is most likely the case.

Shukla and Gough (1979) used concentrated antisera in their experimental work. Based on my experimental evidence, their increase in IgG attachment can be explained solely on the basis of a partial or complete suppression of a competitive inhibitor by changing the IgG attachment from non-specific to specific.

It remains now to see whether the known facts about (1) protein-A, as listed previously; (2) the probable attachment of IgG molecules to untreated grids as discussed earlier; and (3) the attachment of IgG molecules to pA-treated grids, can accommodate the theory that coating grids with pA would not increase the number of possible IgG attachment sites.

When the grid is coated with protein-A, a high proportion of the surface area is occupied by pA, leaving very little space for non-specific attachment. PA is an elongated protein with a theoretical 4, and a practical 2, specific attachment sites for IgG. Thus in the area of the grid occupied by one pA molecule, assuming both attachment sites are on the same side of the pA molecule, only 2 IgG molecules will be attached. We know from theory that there are at least 2 more possible attachment sites for IgG in the area occupied by one pA molecule and there is also the area occupied by a pA molecule that does not have any Fc attachment sites. Thus coating the grid with pA could reduce the capacity of the grid to hold IgG molecules by at least 50%. The results in Table 3/2 show reductions of at least 30%.

It would thus seem as if coating a grid with pA can in fact reduce the number of possible attachment sites for IgG and that this is consistent with the properties of pA.

3.4.1. *Concluding Comments.*

By way of summary of the preceding experimental work and discussion, the following points can be made.

There have been many major and minor modifications to the original concept of IEM as proposed by Derrick (1973b). Some have

kept the method as quick as possible while others have resulted in much longer (up to 18 or 19 hours) techniques.

The experiments described in this section constitute the first account of any detailed comparison of the available methods and their advantages and disadvantages. There are a number of points that have been established by these experiments, and these are:

(1) All methods tested were more sensitive in detecting virus particles in infected plant tissue than using normal negatively stained preparations of sap exudates.

(2) The use of protein-A as developed by Shukla and Gough (1979) is a better technique than the other rapid methods as originally described and against which it was compared at the time.

(3) When the rapid method is used with the coating antiserum at a dilution of 1:1,000, it is equal to the method using pA and in such circumstances the technique of Shukla and Gough is not superior. Not only is the use of protein-A no advantage but it is less economical of antiserum, and has extra grid-handling steps.

(4) Under limiting conditions of virus concentration in samples (a situation in which IEM has its greatest value), the rapid methods are not really adequate, and long virus acquisition times must be used.

(5) The answer to the question "What is the best method to use?", as asked in the introduction, must be that it depends on the virus/host combination from which the samples are taken, and the purpose of the work. From this set of experiments it would appear that the basic method to use would be to coat the grids with antiserum

diluted to ca. 1:1,000 in carbonate buffer for 90 minutes; wash the grids in phosphate buffer; prepare the virus sample in phosphate buffer at the rate of 3 ml per g tissue and vary the acquisition time according to the concentration of virus particles.

There are, of course, more aspects to IEM than those examined in this section and some of them will be looked at in sections 4-7, after which it may well be profitable to look at the answer to the same question again in the light of those findings.

4. THE EFFECT OF IEM ON PARTICLE SIZE DISTRIBUTION OF TOBAMOVIRUSES

"I cannot remember a single first formed hypothesis which had not after a time to be given up or greatly modified."

Charles Darwin.

4.1. Introduction

An important feature used to differentiate viruses is the morphology of the population of virus particles as imaged in the electron microscope. The use of this characteristic was greatly enhanced by the technique of negative staining which was first described by Brenner and Horne (1959). This method makes use of the principle of surrounding or "embedding" the particles within an electron-dense material which dries to a nearly structureless "glass" that supports particles and protects them against the severe distorting forces of drying (Milne, 1972).

The use of morphology as an invaluable aid to the identification of plant viruses, particularly those with elongated particles, was further facilitated by the development of a system of classification based on particle shape and particle length (Brandes and Wetter, 1959).

The combination of leaf dip preparations and particle measurements has been reviewed (Brandes and Bercks, 1965). In their review they discuss the use of a parameter which they call "normal length" as a measurement of the size of a population of virus particles. They define the normal length as the mean of the main maximum of the population and they state it is reproducible to within a range of ca. 10 nm and that it is invariable except for errors resulting from the electron microscope and the technique of measurement. This method of describing virus size has since been widely used in plant virus laboratories but I consider that it has some shortcomings and this point will be taken up in greater detail in the discussion (4.8.).

In spite of claims of the reproducibility of the normal length, variations in the morphology of populations do occur for a

large variety of reasons (e.g. see Edwardson, 1974; Gibbs, Nixon and Woods, 1963; Govier and Woods, 1971; Harris and Westwood, 1964; Lisa and Dellavalle, 1977; Pirie, 1957).

While the use of particle measurement is important in plant virus diagnosis and identification, it can in some instances have the disadvantage that large numbers of electron micrographs may have to be taken to obtain sufficient numbers of particles to measure a population. It is in such a situation that a technique is needed to increase the number of particles present per micrograph and IEM would seem to be an easy, efficient way to obtain this objective.

The use of IEM has been discussed in detail by Milne and Luisoni (1977) and they suggest IEM would be useful for increasing numbers of particles for measurement but go on to state that "an important question in this context is whether the dimensions of virus particles are changed by trapping them on serum-coated grids". They then state that they have found normal lengths unchanged but go on to state "further checks must establish whether the method can be used routinely".

Because of the need to increase particle numbers for measurement, the importance of morphology in virus diagnosis and identification, and the obvious advantages of using IEM to increase numbers of particles, this series of experiments was undertaken to determine whether IEM did affect virus particle shape and size.

4.2. Materials and Methods

In all experiments virus particles were trapped onto grids coated with homologous antiserum diluted 1:1,000 in carbonate buffer. Unless otherwise stated the virus preparations were made using

Fig. 4/1. Ogive showing the percentage of particles less than a given length in populations of 4 Tobamoviruses prepared by negatively staining a crude sap extract from infected leaf tissue.

—■—TOMV
—□— U1-TMV
—△—U2-TMV
—▲— ORSV

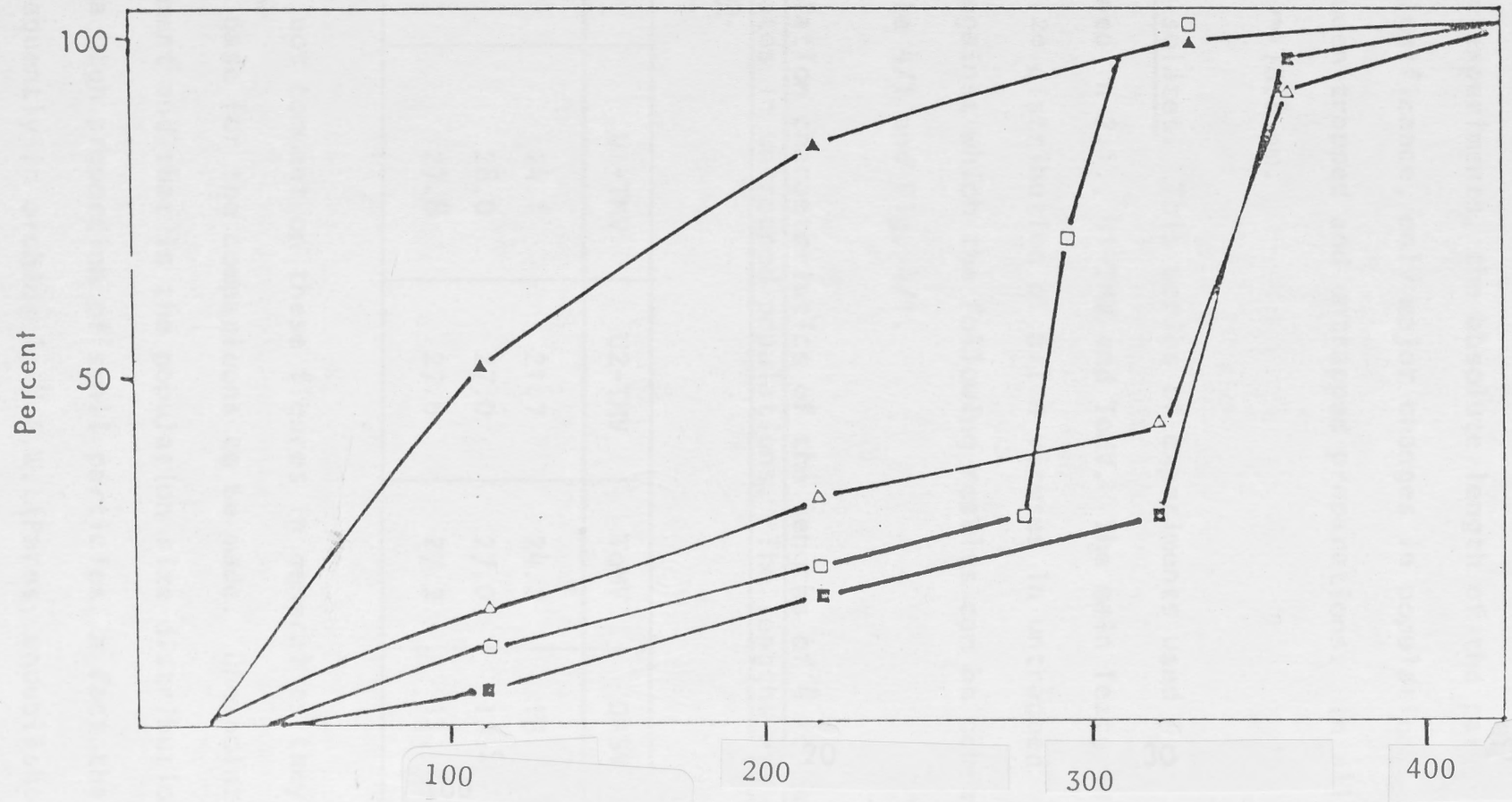


Fig. 4/1.

infected leaf tissue ground in PB at the rate of 3 ml/g infected leaf. Untrapped virus particles were prepared as in 2.3.1. Particle measurements were done as described in 2.3.3. The lengths were not converted to nm and variations in electron microscope magnification were not taken into account between various experiments. This was because, in these experiments, the absolute length of the particles was not of any significance, only major changes in population size distribution between trapped and untrapped preparations. In all experiments 1 mm ~ 10.8 nm.

Virus isolates. This series of experiments used 2 of the 4 viruses mentioned in 2.1., U1-TMV and ToMV. The main features of the population size distribution of all 4 viruses in untrapped populations, and against which the following results can be compared, are shown in Table 4/1 and Fig. 4/1.

Table 4/1. Population characteristics of the lengths of 4 tobamovirus isolates in untrapped populations. The lengths are given in mm.

PARAMETER	U1-TMV	U2-TMV	ToMV	ORSV
Mean length	24.1	21.7	24.2	15.6
Modal length	28.0	27.0	27.0	12.0
Normal length	27.8	27.6	27.2	11.7

I will not comment on these figures in general as they are merely to give a base for the comparisons to be made. One point does require some comment and that is the population size distribution of ORSV, which has a high proportion of small particles, a fact that has been observed frequently in orchids in N.S.W. (Pares, unpublished). Also the description of this virus (Paul, 1975) states that the virus was 300 nm long but there were a lot of small pieces present. The

population used in my studies had a higher percentage of small particles than 300 nm long ones. It appears that in Paul's study the small pieces may have been ignored in subsequent calculations and pre-conceived ideas on Tobamoviruses may have overcome true objectivity. This point will be the subject for later discussion, but it does seem as if the description of ORSV may need some modification.

4.3. The Effect of Trapping on Particle Morphology

Trapping virus particles onto serum-coated grids is a very efficient way to increase particle numbers for measurement and basic to its use for this purpose is the question of whether trapping has any effect on the resulting population size distribution.

In some of my preliminary work on the IEM of ToMV it was noticed that there regularly appeared to be a higher proportion of small particles than is usually seen in untrapped preparations of this virus. The fact that it was so noticeable suggested that the proportion of small particles must have been considerably more than usual and preliminary measurements revealed only 24% of the particles were in the region of the 300 nm peak in the trapped population, the majority of the particles being much smaller than 300 nm. The appearance of these trapped and untrapped populations is shown in Fig. 4/2.

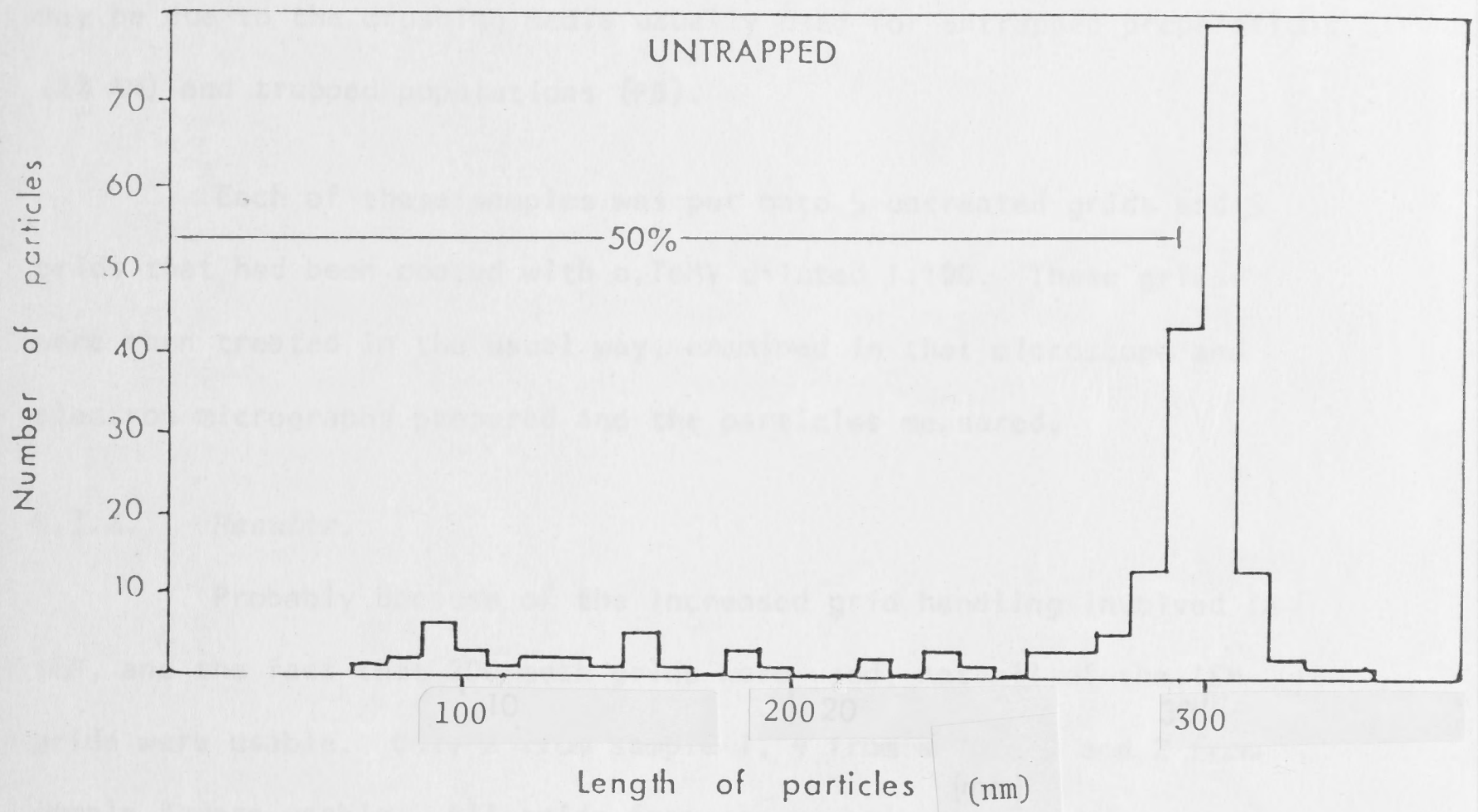
Such observations indicated that an investigation should be made on the effect of trapping on population size distribution.

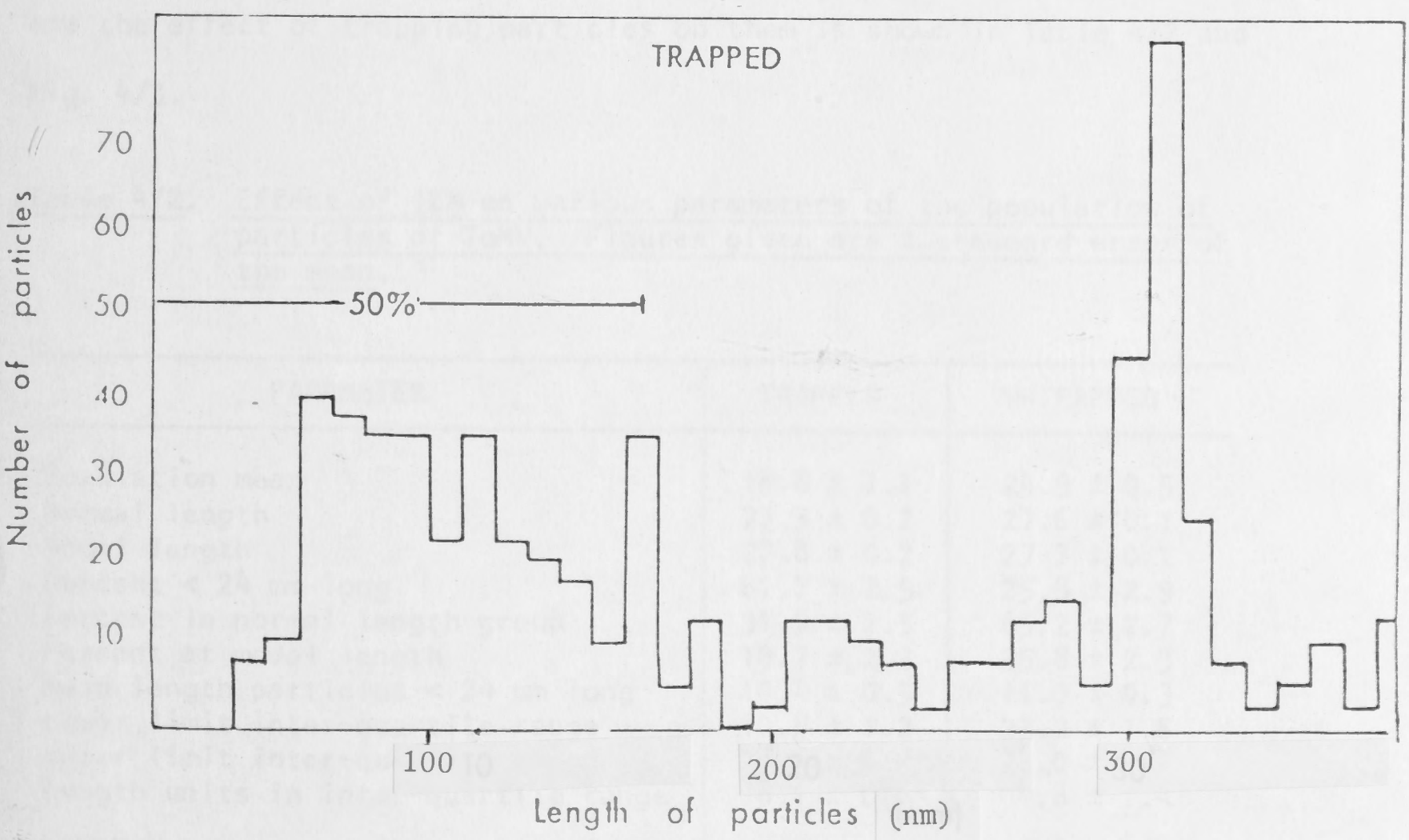
4.3.1. *Materials and Methods.*

Three separate tomato plants, each infected with ToMV were selected (Sample Nos. 1, 2, 3) and pieces of leaf tissue were taken from each and crushed in 2% ammonium molybdate (2% AM) at the rate of 3 ml per g leaf tissue. The reason for using 2% AM and not PB to

Fig. 4/2. Histograms illustrating differences in size range of particles of ToMV trapped on anti-serum-coated grids compared to untrapped populations. The particle lengths are given in ngs and 1 nm ≈ 10.8 nm.

Fig. 4/2.


UNTRAPPED
70
60
50%
50
40
30
20
10
Number of particles
100
200
300
Length of particles (nm)


TRAPPED
70
60
50%
50
40
30
20
10
Number of particles
100
200
300
Length of particles (nm)

crush the leaf tissue was to overcome any effect on particle size that may be due to the crushing media usually used for untrapped preparations (2% AM) and trapped populations (PB).

Each of these samples was put onto 5 untreated grids and 5 grids that had been coated with a.ToMV diluted 1:100. These grids were then treated in the usual way, examined in that microscope and electron micrographs prepared and the particles measured.

4.3.2. *Results.*

Probably because of the increased grid handling involved in IEM, and the fact that 200 mesh grids were used, not all of the IEM grids were usable. Only 2 from sample 1, 4 from sample 2 and 2 from sample 3 were usable. All grids from the negatively stained preparations were satisfactory.

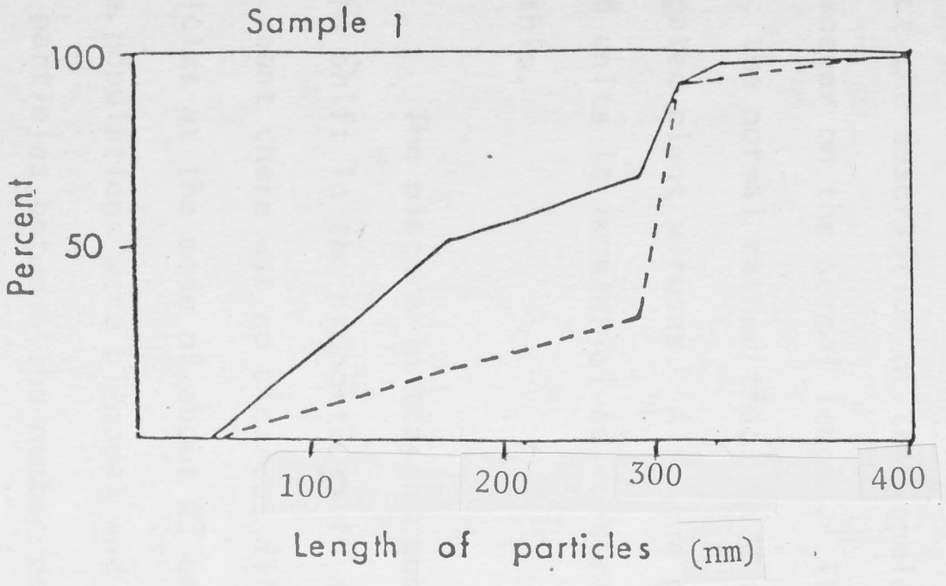
In all 10 characteristics of the populations were evaluated and the effect of trapping particles on them is shown in Table 4/2 and Fig. 4/3.

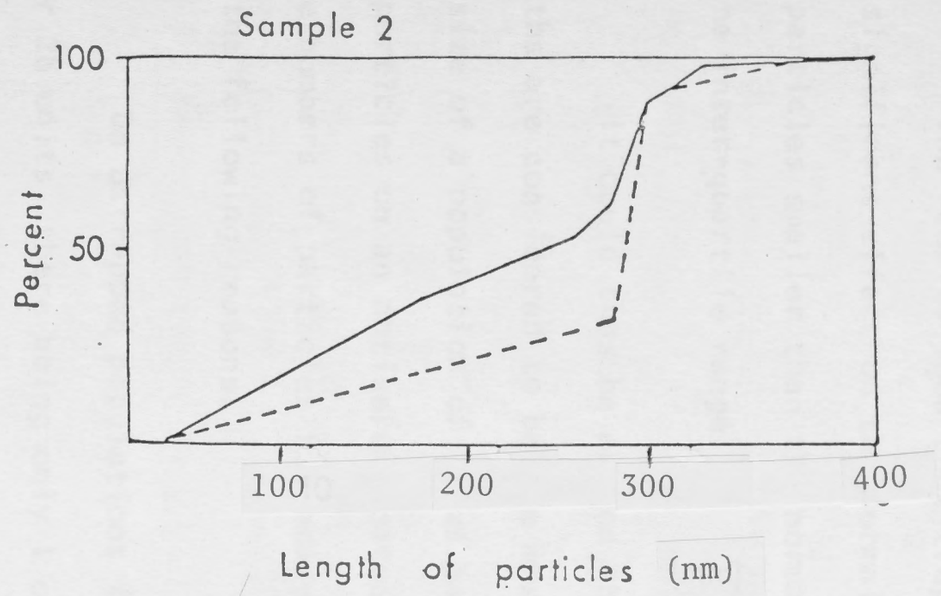
Table 4/2. Effect of IEM on various parameters of the population of particles of ToMV. Figures given are ± standard error of the mean.

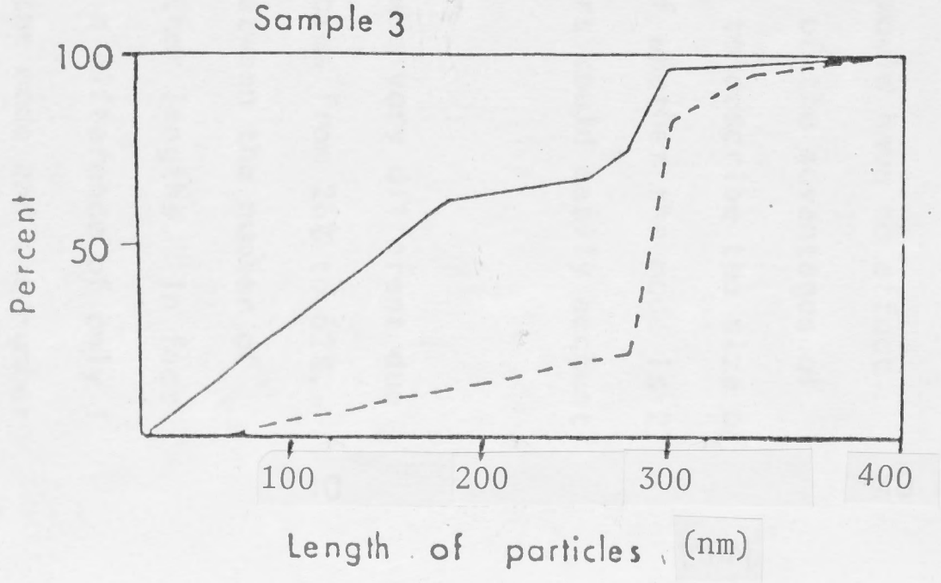
PARAMETER	TRAPPED	UNTRAPPED
Population mean	18.8 ± 1.1	24.9 ± 0.5
Normal length	27.3 ± 0.2	27.6 ± 0.1
Modal length	27.8 ± 0.2	27.7 ± 0.1
Percent < 24 mm long	61.7 ± 2.9	25.8 ± 2.9
Percent in normal length group	31.5 ± 2.5	65.2 ± 2.7
Percent at modal length	18.7 ± 2.3	35.8 ± 2.3
Mean length particles < 24 mm long	10.4 ± 0.5	11.0 ± 0.3
Lower limit inter-quartile range	10.8 ± 1.2	23.2 ± 1.5
Upper limit inter-quartile range	26.9 ± 0.7	28.0 ± 0.2
Length units in inter-quartile range	16.1 ± 0.6	4.8 ± 1.4

Fig. 4/3. Ogives showing the consistent effect of IEM in changing the size distribution of particles of ToMV. For the IEM preparations samples 1 and 3 were the mean of two grids per sample and sample 2 was the mean of 4 grids. All the untrapped samples were the mean of 5 grids per sample.

—— TRAPPED
---- UNTRAPPED


Sample 1
100
50
Percent
100
200
300
400
Length of particles (nm)


Sample 2
100
50
Percent
100
200
300
400
Length of particles (nm)


Sample 3
100
50
Percent
100
200
300
400
Length of particles (nm)

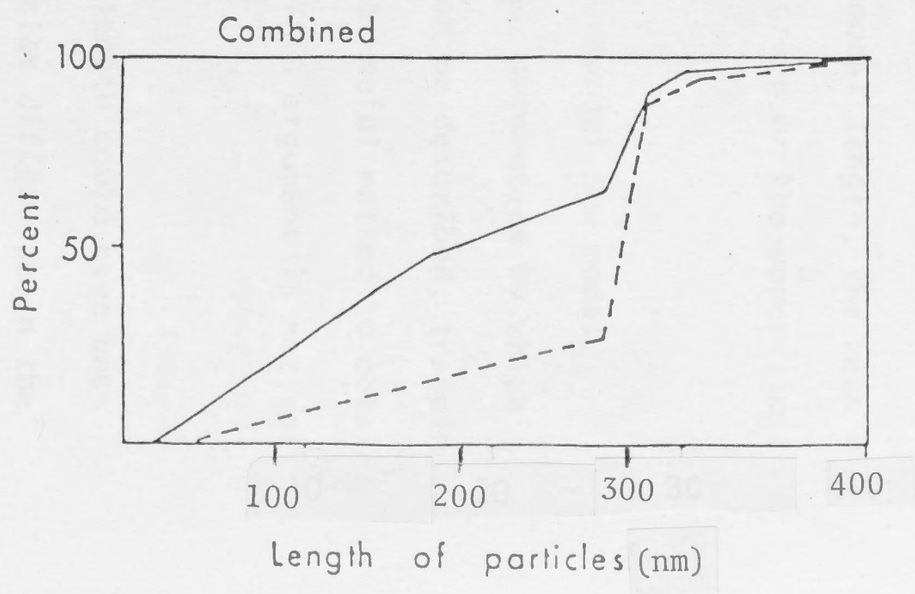

Combined
100
50
Percent
100
200
300
400
Length of particles (nm)

Fig. 4/3.

4.3.3. *Discussion.*

The results show that trapping virus particles did not have any significant effect on the normal length, modal length, the mean of the particles smaller than the normal length group or the upper limit of the inter-quartile range.

It could thus be argued that, as the normal and modal lengths are considered to be the most important parameters by which the size of a population of virus particles can be described, trapping the particles on an antiserum-coated grid is a useful method to obtain large numbers of particles for measurement. This argument is not valid for the following reasons.

On untrapped populations the modal length could have been 27 or 28 units, there being only 1 or 2 particles difference in the number present in each. Another set of measurements could easily change the mode from one to the other. This does not, however, really affect the description of the population and would have no affect whatsoever on the normal length. This is one of the advantages of using the normal rather than the modal length to describe the size of elongated plant viruses. Also the question of whether the mode is 27 or 28 units is immaterial as measurement errors could easily account for this.

The picture in trapped populations was very different due to the shift in the proportion of small particles from 26% to 62%. This meant there was no clearcut difference between the number of particles at the mode of about 28 units and other lengths. In fact these populations were bi-modal and there was a difference of only 1 or 2 particles between the number present at the mode and the number present at the secondary mode. The difference in this instance could

not be accounted for by measurement errors but the mode could easily be shifted drastically if other sets of particles were measured.

As pointed out previously such variations do not affect the normal length of untrapped populations but would cause a large change in the normal length of trapped populations. In fact in some populations examined during the course of this research there have been populations in which the main mode was in the region of the smaller particles.

Some populations of Tobamoviruses do in fact have bi-modal populations when untrapped particles are measured (e.g. potato mop-top virus). These occurrences bring up the question of validity of using a single criterion, particularly the normal length, to describe the length of elongated plant viruses and this will be expanded in the general discussion (4.8).

It would seem from these studies that great care should be exercised when using IEM to obtain large numbers of particles for the purpose of length measurements. It would, in fact, seem undesirable to do so.

4.4. The Effect of different IEM methods on particle size distribution

Section 4.3 described an experiment proving that IEM did in fact alter the characteristics of the observed particle population of ToMV and the major effect was an increase in the proportion of small particles. There have been a number of methods of IEM described and there are some key differences in some of these methods.

In the light of the results of the previous experiment it is necessary to know whether the buffer used in antiserum dilution had any effect and whether rapid and slow methods have similer effects.

4.4.1. *Materials and Methods.*

Three methods were chosen for comparison: (1) a rapid method (Milne and Luisoni, 1977); (2) a slow method using PB to dilute the antiserum to 1:1,000 (Roberts and Harrison, 1979); and (3) a slower method using CB as the antiserum diluent (Plumb, pers. comm.). In this section these methods will be designated (1) rapid, (2) slow and (3) slowest.

In the previous section the slow method was used and the virus was ToMV. As this method and virus was examined in detail in that section it was not repeated here but used in the comparisons. The slowest method was done using U1-TMV and the rapid one using ToMV.

4.4.2. *Results.*

Both the rapid and the slowest methods caused an alteration of the population similar to the change described previously for the slow method. In the case of the slowest method on virus U1-TMV the population had two peaks, one at about 7 units and one at 28 units of length. With the rapid method and virus ToMV the peak at about 7 units was present but smaller than that for U1-TMV.

These results are summarised in Table 4/3 and Fig. 4/4.

Table 4/3. Effect of three different IEM methods on particle size.

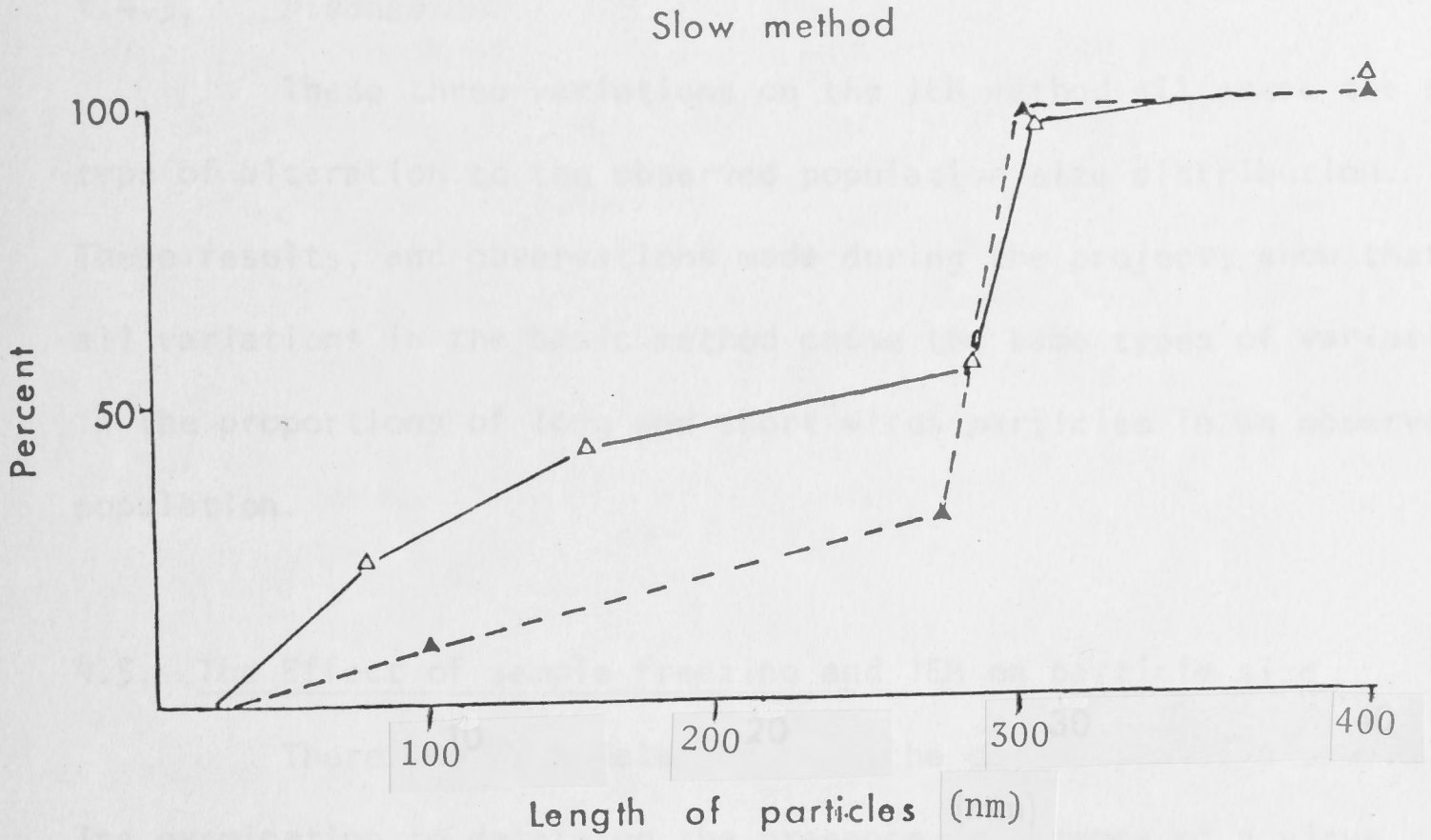
PARAMETER	METHOD AND VIRUS		
	SLOW ToMV	SLOWEST U1-TMV	RAPID ToMV
Mean	18.8	19.3	16.3
Mode	28	28	28
Normal length (NL)	27.3	28.3	28.3
Percent in NL peak	31.5	49	31.5
Percent < NL peak	61.7	48	65
% at Modal Length	18.7	23	19

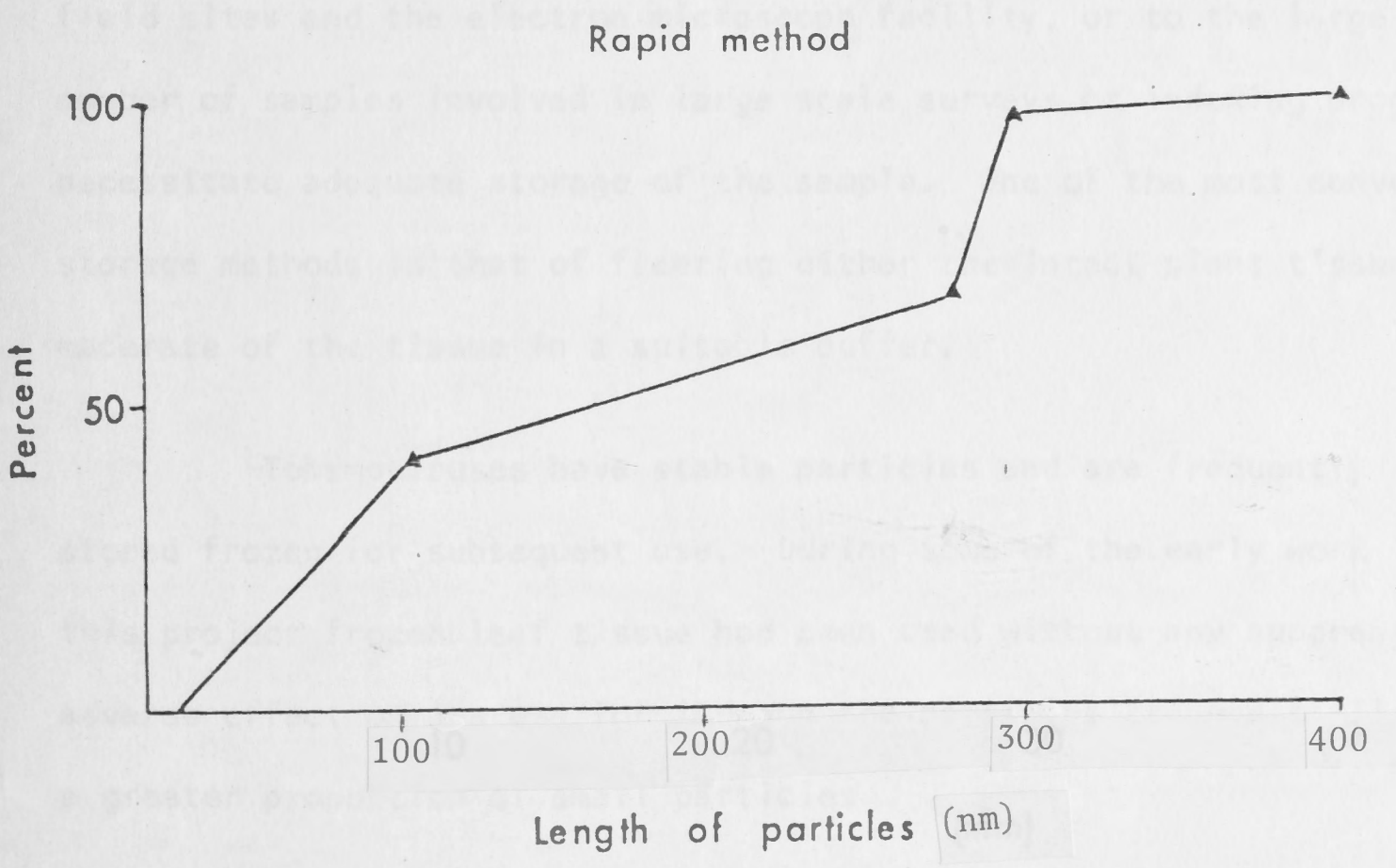
Fig. 4/4. Ogives to show that IEM causes an increase in the proportion of small particles whether the IEM method used is rapid or slow.

—— △ —— Trapped

- - - ▲ - - - Untrapped

Fig. 4/4.


Slow method
100
50
Percent
100
200
300
400
Length of particles (nm)


Rapid method
100
50
Percent
100
200
300
400
Length of particles (nm)

4.4.3. *Discussion.*

These three variations on the IEM method all cause the same type of alteration to the observed population size distribution. These results, and observations made during the project, show that all variations in the basic method cause the same types of variations in the proportions of long and short virus particles in an observed population.

4.5. The Effect of sample freezing and IEM on particle size

There may be a delay between the collection of a sample and its examination to determine the presence or absence of a virus infection. There are many reasons for such a delay being an unavoidable and inherent part of the process of virus diagnosis or large scale surveys of virus infections.

Such delays, whether they be due to the distance between the field sites and the electron microscope facility, or to the large number of samples involved in large scale surveys or indexing programs, necessitate adequate storage of the sample. One of the most convenient storage methods is that of freezing either the intact plant tissue or a macerate of the tissue in a suitable buffer.

Tobamoviruses have stable particles and are frequently stored frozen for subsequent use. During some of the early work in this project frozen leaf tissue had been used without any apparent adverse effect on its use for IEM and the particles trapped still had a greater proportion of small particles.

It was decided that this aspect should be examined in greater detail and to concentrate on storage in the frozen state after maceration in buffer.

4.5.1. *Materials and Methods.*

Tomato leaf tissue, infected with ToMV, was used and the macerate was divided into 8 portions and frozen at -20°C. Every 2 weeks one of these portions was prepared for electron microscopy and measurement by the rapid technique. Thus samples were examined after being frozen for 2, 4, 6, 8, 10, 12, 14 and 16 weeks. Particles of trapped and untrapped populations were measured and the population characteristics were compared.

4.5.2. *Results.*

The results are shown in Table 4/4 and Figs 4/5 and 4/6. There did not appear to be any variation of the parameters with time of freezing, as can be seen in Fig. 4/6. The overall means of the 8 samples are used for Table 4/4 and Fig. 4/5.

Table 4/4. Effect of trapping on virus particles from frozen macerate. Figures are the overall means for the 8 samples ± standard error of the mean.

PARAMETER	TRAPPED	NOT TRAPPED
Mean	20.1 ± 0.35	23.4 ± 0,5
Mode	28.4 ± 0.18	28.3 ± 0.33
Normal length	28.3 ± 0.08	28.5 ± 0.21
% in Normal length group	44.2 ± 1.67	51.2 ± 0.91
% < Normal length group	50.9 ± 1.59	39.8 ± 2.09
% at mode	19.7 ± 1.08	23.3 ± 1.67
Inter-quartile range	18.5 ± 0.38	12.0 ± 1.48

4.5.3. *Discussion.*

The results show that storage of infected sap at -20°C does not appreciably affect either the population size distribution of particles when examined untrapped or the change in size distribution observed when the particles are trapped.

It has been shown (Sehgal and Das, 1975) that a freeze/thaw cycle can substantially affect sedimentation rate, serological activity

Fig. 4/5. Ogive showing the effect on population size distribution of trapping virus particles from infected plant sap that has been frozen at -20°C. are compared. The particle lengths are given in mm and 1 mm ~ 10.8 mm.

—△— Trapped

- - ▲ - - Untrapped

Fig. 4/5.

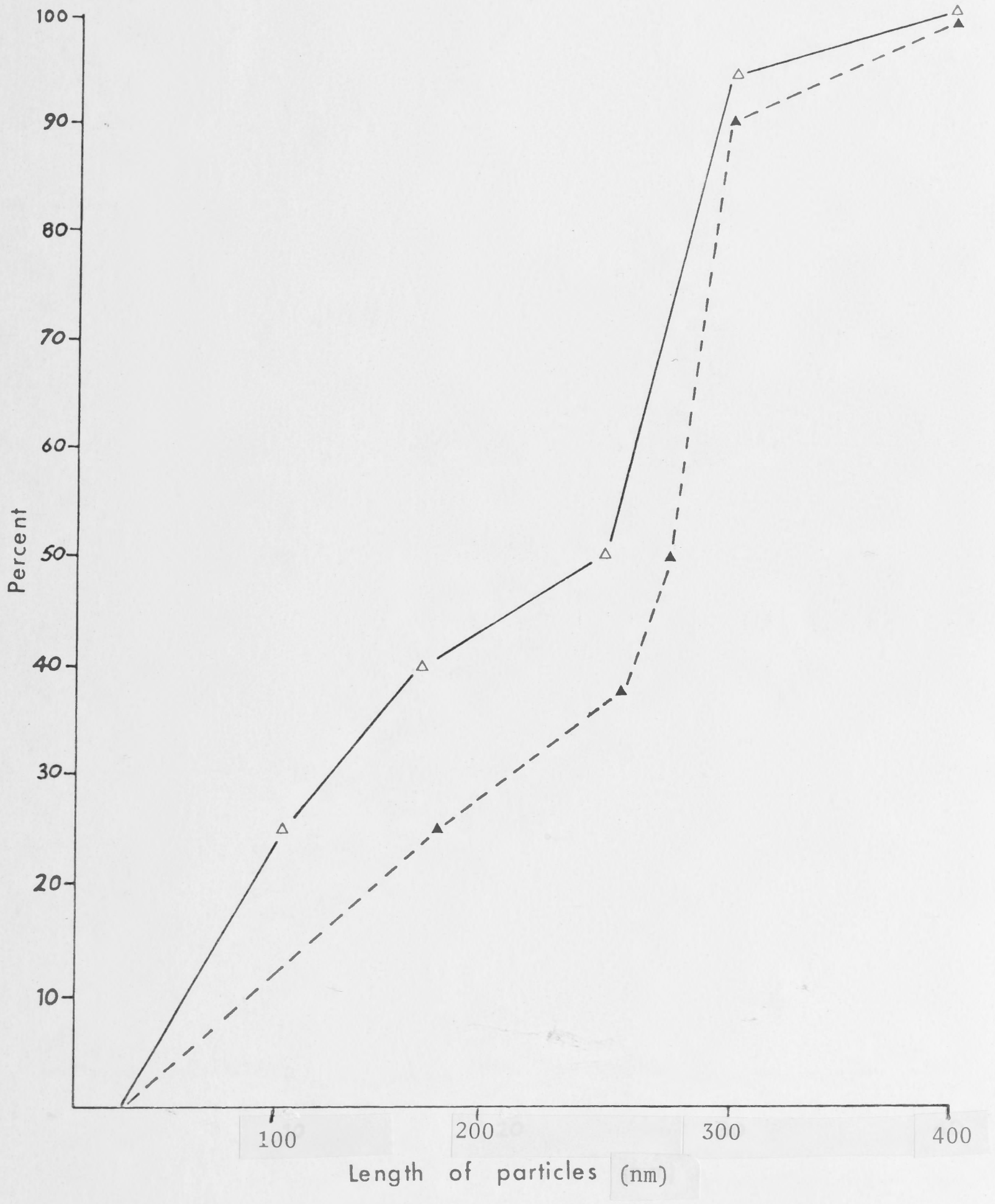

100
90
80
70
60
50
40
30
20
10
Percent
100
200
300
400
Length of particles (nm)

Fig. 4/6. These graphs show the effect of IEM on leaf tissue infected with ToMV and macerated in phosphate buffer before freezing at -20°C.

TRAPPED ————————

UNTRAPPED - - - - -

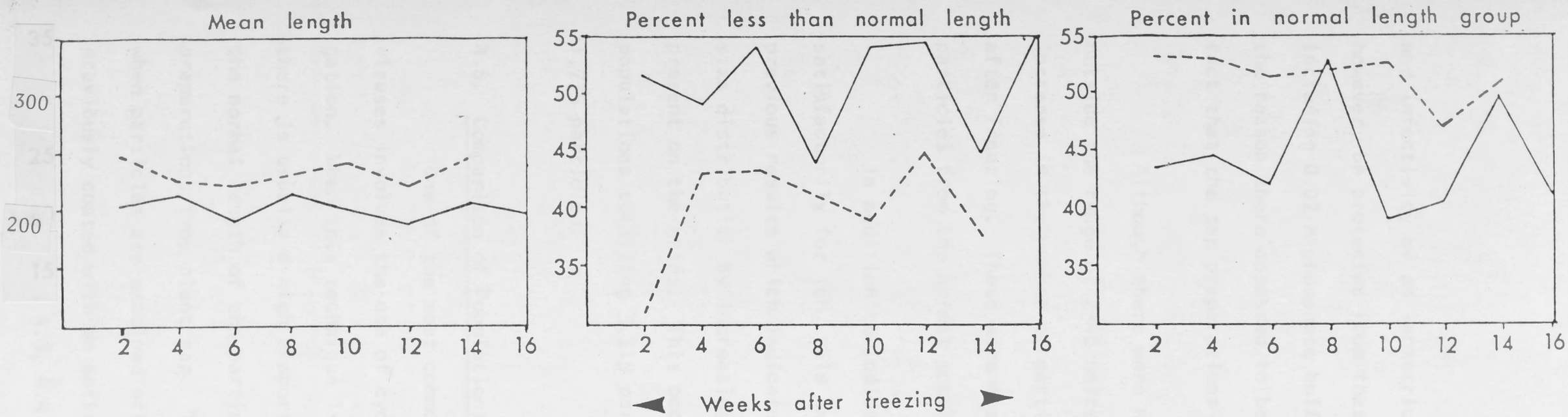

Mean length
300
200
Percent less than normal length
55
50
45
40
35
Percent in normal length group
55
50
45
40
35
2 4 6 8 10 12 14 16
Weeks after freezing
Percent at mode
30
25
20
15
10
Inter-quartile range
200
100
2 4 6 8 10 12 14 16
Weeks after freezing

Fig. 4/6.

and infectivity of an isometric plant virus. The particles could, however, be protected from these effects by a number of chemicals including 0.02 M phosphate buffer (pH 7.0). It is thus possible that the reason there appeared to be no change with freezing was due to the fact that the sap preparations were made in phosphate buffer.

Although there were no detailed counts made of particles outside the range of 2-40 units in length there did appear to be an increase in the number of particles that were much larger than 40 units after freezing. These were most probably end-to-end aggregates of particles from the normal population.

In addition to indicating that frozen sap could be used satisfactorily for IEM, this experiment provided data to reinforce previous results which indicate that IEM does alter the population size distribution by increasing the proportion of small particles present on the grids. This occurred consistently over the 8 trapped populations totalling 3,319 particles and 6 untrapped totalling 1,775 particles.

4.6. Comparison of Populations after purification and after IEM

One of the most commonly used methods to purify plant viruses involves the use of cycles of low and high speed centrifugation. When this technique is used on rod-shaped plant viruses there is usually a high proportion of particles that are less than the normal length of the particles seen in negatively stained preparations from plant sap. This is similar to the effect seen when particles are examined after being put onto grids that have been previously coated with an antiserum specific to the virus, as described in sections 4.3, 4.4 and 4.5.

This experiment was therefore set up to compare populations of particles present in (a) negatively stained sap on a grid not coated with antiserum (b) negatively stained sap on a grid previously coated with antiserum and (c) a resuspended high speed pellet on a grid not coated with antiserum.

4.6.1. *Materials and Methods.*

Particles of U1-TMV were purified from infected tobacco leaves as described in the purification schedule in section 2.1.4. The resuspended third high-speed pellet was then diluted 1:100, mixed 1:1 with 2% A.M., put onto a grid, dried and used for electron microscopy.

Some of the original infected leaf tissue was macerated in 0.06 M phosphate buffer (3 ml/g) and put onto grids, one of which had not been coated with antiserum and one that had been treated with a.U1-TMV diluted 1:1,000.

4.6.2. *Results.*

The results are shown in Fig. 4/7. The proportion of small particles was much higher in the purified preparation than in either of the other two preparations. In the purified sample 64% were in the range 3-14 units with a mode (10% of particles) at 5 units. The calculated normal length for this population was 7.6 units.

4.6.3. *Discussion.*

There is more than one explanation for the increase in proportion of small particles after the two treatments of (a) trapping on antiserum-coated grids and (b) purification by high speed centrifugation. This aspect will be examined in greater detail in a discussion of the general topic of altered observed populations (4.8).

Fig. 4/7. Ogive comparing a trapped population of U1-TMV and untrapped populations from crude sap and a high speed pellet from a purification run using differential centrifugation. Note that high speed pelletting caused greater disruption than IEM.

---▲---Untrapped
——△——Trapped
---●---Untrapped pellet

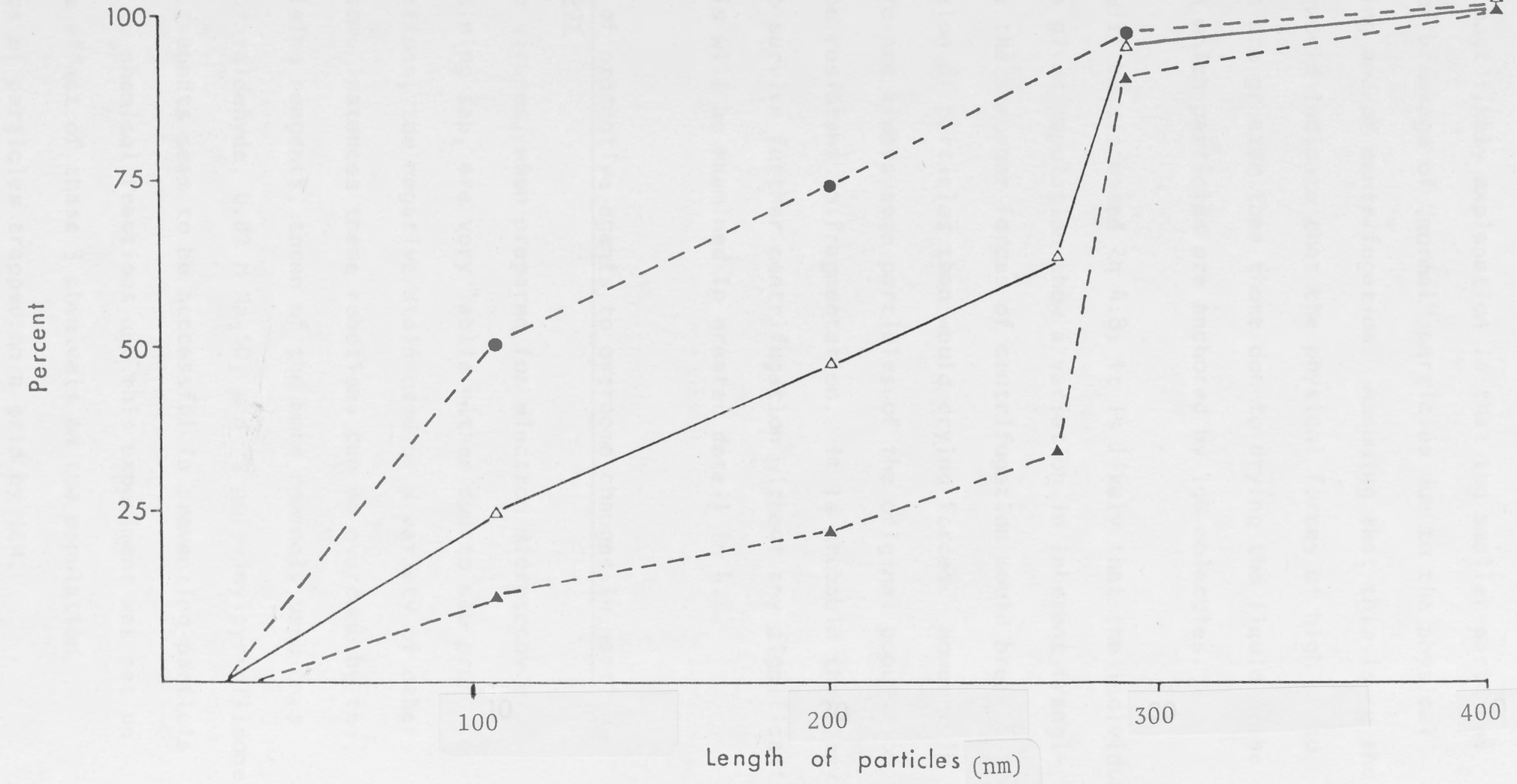


Fig. 4/7.

The most likely explanation is that the smaller particles are products of breakage of "normal" particles due to the physical forces of drying and/or centrifugation. Assuming that this is so the results above would indicate that the physical forces of high speed centrifugation are greater than those due to drying the liquid phase from a grid on which particles are anchored by IgG molecules.

As will be discussed in 4.8, it is likely that the individual particles in a given population show a variation in inherent frangibility. Hence the stronger forces of centrifugation would break a larger proportion of particles than would drying forces. However in all cases there are always some particles of the original population that seem to be resistant to fragmentation. It is probable that they may be able to survive further centrifugation without any significant breakage. This will be examined in greater detail in 4.8.

4.7. The use of protective agents to overcome changes in particle morphology

Some viruses, when prepared for electron microscopy by negatively staining sap, are very labile either due to sap prone to oxidation reactions, the negative stain used or a variety of other reasons. In some instances these reactions can be overcome by the use of stabilising reagents, three of the more commonly used ones being 2.5% glutaraldehyde, 0.02 M Na_2SO_3 and 2% polyvinylpyrrollidone (PVP). These reagents seem to be successful in preventing particle breakdown due to chemical reactions and this experiment was set up to examine the effect of these 3 chemicals on the population characteristics of particles trapped on a grid by IEM.

4.7.1. *Materials and Methods.*

Each of the protective agents was incorporated, at the

appropriate concentration, into the 0.06 M phosphate buffer used to macerate infected leaf tissue. Samples macerated in phosphate buffer were used as controls. In all instances samples were put onto grids precoated with antiserum and grids that had not been so treated.

4.7.2. *Results.*

It was clear from examination in the microscope, before measurements were done, that the protective agents had no effect on the particles in so far as preventing the increase in the proportion of smaller particles. Three representative populations are shown in Fig. 4/8.

4.7.3. *Discussion.*

The two chemicals Na_2SO_3 and PVP are useful in protecting viruses against chemical breakdown from sap that is prone to oxidation (e.g. sap from Rosaceae, grapevines and poplar) and in the case of labile viruses (Milne, 1980). Glutaraldehyde is of particular value as a fixative when used to protect viruses from disruption that can occur due to a negative stain such as PTA (Gibbs et al., 1963).

The 3 protective agents are effective against a range of chemical effects on viruses, and in this instance did not prevent an increase in the proportion of small particles in trapped virus populations. Because of this it seems certain that the increase in the number of observed small particles is not due to any of the more common chemical reactions that can occur in negatively stained plant sap preparations. It also seems unlikely that the phenomenon is due to a chemical reaction because of the apparent general ineffectiveness of these 3 agents.

It therefore seems most likely that the small particles originate from the larger particles by breakage due to the physical

forces of drying or that they are always present but are preferentially attracted to the antiserum coating on the grids. This point will be taken up in the general discussion of this section (4.8).

If, in fact, the particles do come from physical breaking of the large particles then it would appear that these chemicals do not have any effect in strengthening the particles and thus do not protect them from such physical forces.

4.8. General Discussion

4.8.1. *Published sizes of Tobamoviruses.*

Before the importance and implications of these results are discussed it is appropriate to examine the Tobamovirus group in relation to the known facts about their particle sizes. The particle lengths of various members of the group are shown in Table 4/5, which is based on the series of "Descriptions of Plant Viruses" published by the CMI/AAB and the bulletin numbers from which the sizes were obtained are shown in the table.

These figures indicate that the members of the tobamovirus group can be placed into 3 categories based on particle length descriptions, but probably there are only two categories, if the facts are examined more thoroughly. Based on the published descriptions the 3 groups would be (a) those that have the majority of particles 300 nm long: tobacco mosaic virus; ribgrass mosaic; cucumber green mottle mosaic; tomato mosaic. (b) those that are described as having the particle length 300 nm but which the authors state have a high proportion of small particles: Sunn-hemp mosaic; Odontoglossum ringspot. (c) those described as having more than one mode of particle length: soil-borne wheat mosaic; potato mop-top; beet necrotic yellow vein.

It would also seem from Table 4/5 that all the definitive members have a unimodal population size distribution and this is also misleading as will be shown by further examination of the facts of two of these viruses: Sunn-hemp mosaic (SHMV) and Odontoglossum ringspot (ORSV).

Table 4/5. Sizes of some members of the tobamovirus group.

CMI/AAB Bull. No.	VIRUS	LENGTH (nm)
	DEFINITIVE MEMBERS (a)	
151	Tobacco mosaic	300
152	Ribgrass mosaic	300
154	Cucumber green mottle mosaic	300
156	Tomato mosaic	300
153	Sunn-hemp mosaic	300 } (b)
155	Odontoglossum ringspot	300 } (b)
	TENTATIVE MEMBERS	
77	Soil-borne wheat mosaic	300; 110-160
138	Potato mop-top	250-300; 100-150
144	Beet necrotic yellow vein	390; 270; 65-105

These figures are based on those given in the CMI/AAB "Descriptions of Plant Viruses" series of bulletins.

(a) : The classification into definitive and tentative members based on Gibbs (1977).

(b) : The descriptions of these two viruses state that the preparations always contain many short particles.

4.8.2. *The particle size distributions of ORSV and SHMV.*

One of the early studies on SHMV (Kassanis and McCarthy, 1967) indicated that all the isolates examined had a majority of particles less than 300 nm long, that the proportion of small particles varied with the strain and, in at least one strain, the proportion of small particles varied with the temperature at which infected plants were grown. If an examination is made of more recently published

histograms of this virus it can be seen that, if all particles are considered, the mode and normal length are in the region of 40 nm (Bruening et al., 1976) or 50 nm (Whitfeld and Higgins, 1976). It thus seems that the only reason this virus is not considered to be multiparticulate is that, in the original descriptions of particle size, it has been considered advisable to ignore the smaller particles because of some preconceived notion the author(s) had about Tobamoviruses.

In describing ORSV, Paul (1975) stated that the length was 300 nm but conceded that preparations contained many short particles and "discs". It appears these weren't considered when the particle size calculations were made. When NSW isolates of ORSV were examined in this and previous (Pares, unpub.) studies, and all particles were considered, the modal length was about 100 nm, normal length 98 nm, and the population mean was 146 nm. Approximately 75% of the population was less than 200 nm long.

Thus in the case of both of these viruses it is only possible to say the virus has particles 300 nm long when the small particles are not considered. It would be better to describe the particle size of both these viruses by saying that they are multiparticulate, SHMV having particles of two predominant lengths, 40 and 300 nm, and ORSV having 100 and 300 nm as the two predominant lengths.

These facts show that bimodal populations do occur in both the definitive and tentative members, even though the published descriptions do not seem to indicate it. It seems from these facts that the description of the population of particles depends, at least in part, on the authors concerned and on their adherence to a criterion such as normal length as a means of describing a virus size. In fact

the concept of normal length, which was proposed before bimodal populations of elongated viruses had been described, should perhaps be discontinued or used only with caution. By definition, it assumes a unimodal size distribution for all viruses and it therefore encourages the practice of ignoring other peaks that may occur in a size-distribution.

There are, in fact, no really clear guidelines as to how population sizes of viruses should be described. Indeed there should be no need to set out guidelines for such an apparently simple operation, as it would not be expected that data would be subject to the prejudicial interpretation that evidently occurs.

4.8.3. *The significance of the small particles.*

A question that arises from this is whether the small particles that occur in negatively stained sap preparations, that have not been subjected to any other preparative procedures other than the negative staining, have any significance or function in an infection of a susceptible plant. There have been a number of detailed studies of this using Tobamoviruses, almost all of them being done on SHMV. This virus was chosen because of the greater quanity of small particles that can be obtained for experimental work.

One of the earliest reports of work on the multiparticulate nature of SHMV was done using a number of different isolates (Kassanis and McCarthy, 1967) and they concluded that all isolates had a majority of particles less than 300 nm long and the proportion of them varied with the strain and temperature at which they were propagated.

Subsequent studies (Whitfeld and Higgins, 1976) showed the ratios varied from 1:1 to 2:1 (small:large), the short (S) particles

Fig. 4/8. Ogive comparing trapped and non-trapped populations when glutaraldehyde was incorporated in the phosphate buffer. The other protective agents used produced similar results.

——△——Trapped

- - -▲- - -Untrapped

——○——Ga Trapped

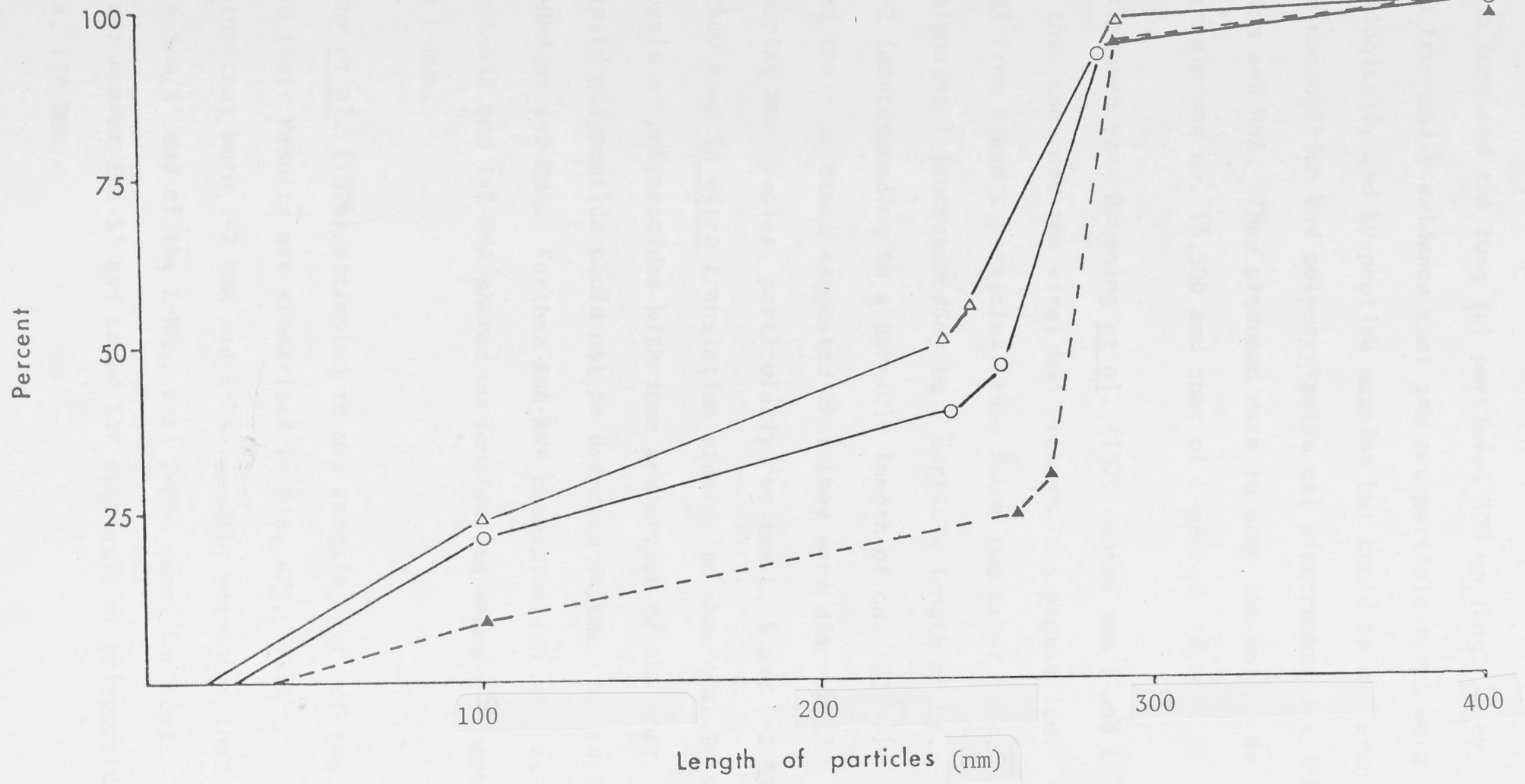


Fig. 4/8.

being ca. 40 nm long and the long (L) particles 300 nm long. They also concluded from their evidence that the two particle types were identical serologically and by peptide mapping but could be differentiated by base composition and polyacrylamide gel electrophoresis (PAGE) of both proteins and RNA. They produced data to show the molecular weight of S-protein was ca. 16,500 and that of L-protein 18,000.

In the same year Bruening et al. (1976) noted the S and L particles and then examined the viral RNA present in a population. In addition to RNA from S and L particles, they found two other classes which they designated I (corresponding to a particle length of ca. 210 nm) and I-2 (corresponding to a particle length of ca. 100 nm). They considered their evidence suggested that they were discrete, naturally occurring RNA species, particularly for the L, S and I-2 RNA. Additionally they used in vitro translation studies to show that S-RNA directed synthesis of polypeptides with some properties of the coat protein but capsid polypeptide could not be detected in the translation products of L-RNA or I-2 RNA. Further RNA-RNA hybridisation studies indicated that S-RNA and I-2 RNA shared nucleotide sequences with each other and with L-RNA.

Beachy et al. (1976) attempted to map certain parts of the RNA of SHMV and their results are summarised in Fig. 4/9. This diagram indicates that both I-2 RNA and S-RNA contain sequences that are shared with the 3' end of the L-RNA, that S-RNA codes for coat protein and areas nearer the 5' end coded for synthesis of polypeptides with a MW of ca. 130,000.

These results were supported by further detailed studies of synthesised polypeptides analysed by a variety of techniques (Higgins, et al., 1976).

Fig. 4/9. Map of TMV showing probable locations of S-RNA, I.2-RNA and L-RNA in a particle based on information in Beachy et al. (1976), Fig. 5 : 506.

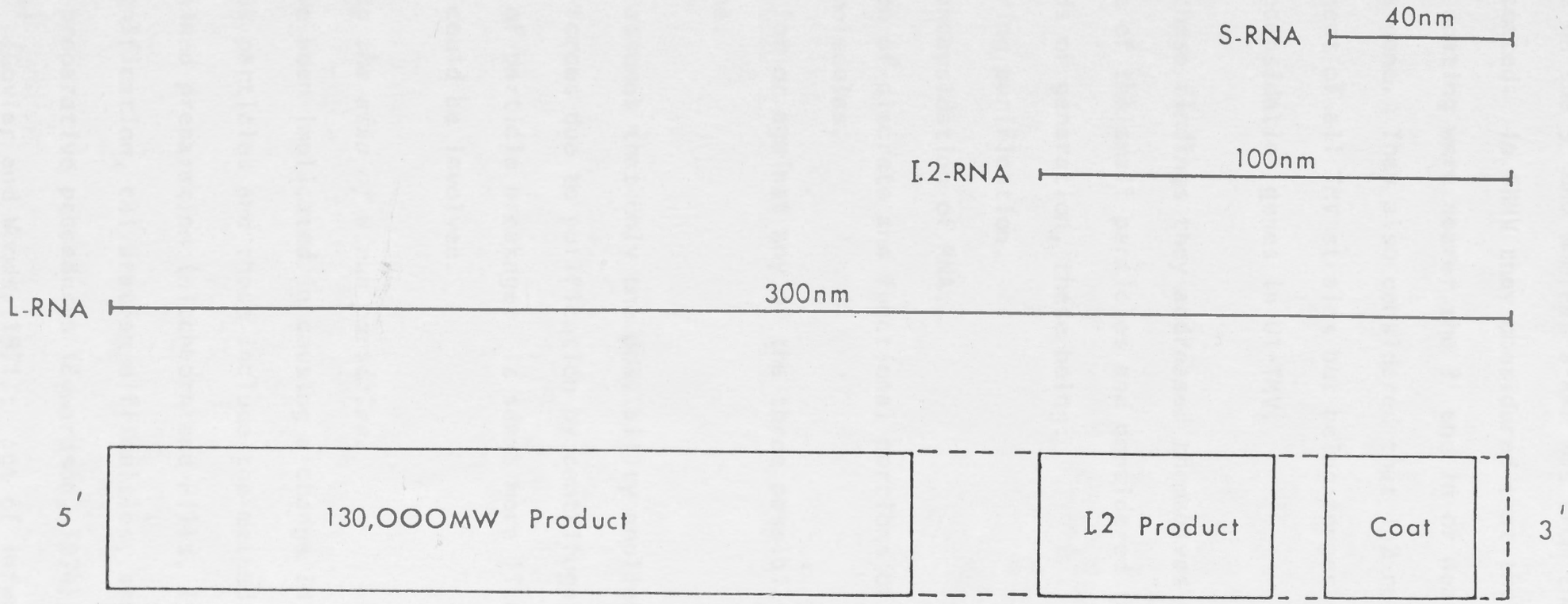

S-RNA
40nm
I.2-RNA
100nm
L-RNA
300nm
5'
130,OOOMW Product
I.2 Product
Coat
3'

Fig. 4/9.

Beachy and Zaitlin (1977) assumed that S-RNA was present in all strains of tobamoviruses and that in U1-TMV the recognition sequences for coating were on the 5' end whereas S-RNA was from the 3' end and therefore not coated. In SHMV they considered that the recognition sequences for coating were nearer the 3' end in or near the coat protein synthesis gene. They also considered that 1-2 rods represent a genuine component of all TMV strains but being longer than S rods they include the encapsidation genes in U1-TMV.

As a result of these findings they addressed themselves to the question of the source of the small particles and considered there were three possible methods of generation, these being:

(1) Breakage during purification.

(2) Incomplete encapsidation of RNA.

(3) Encapsidation of discrete and functional portions of virion RNA molecules.

Without any hard evidence for or against any of the three possibilities they favoured the third one.

Such a decision assumes that only one possibility applies which is most unlikely as forces due to purification by centrifugation are well-known as a cause of particle breakage. It seems more likely that more than one factor could be involved.

4.8.4. *Factors affecting the size of virus particles.*

Many factors have been implicated in causing a change in morphology or size of virus particles and these include the method of preparing a negatively stained preparation (Hitchborn and Hills, 1965); fluctuating microscope magnification, calibration difficulties, strain variation, host plant and preparative procedures (Edwardson, 1974); presence or absence of Mg^{++} (Govier and Woods, 1971); age of infection

(Brandes and Bercks, 1965); the use of potassium phosphotungstate (KPTA) as a negative stain on a number of different viruses such as Vaccinia virus (Harris and Westwood, 1964), rod-shaped plant viruses (Gibbs et al., 1963), alfalfa mosaic (Gibbs et al., 1963), and potexviruses (Lisa and Dellavalle, 1977). Part of the effect of the negative stain could be due to electrostatic charge effects caused by the KPTA polyanion adsorbing to the surface (Horne, 1967b).

Plant proteolytic enzymes can also play an important part in the degradation of virus particles (Shepard and Secor, 1972; Tremaine and Agrawal, 1972; Lesemann and Koenig, 1978).

4.8.5. *The stability of tobamoviruses.*

It can thus be seen from a brief summary of the literature that a wide range of physical and chemical factors have been implicated. The tobamovirus particles are generally regarded as very stable, rigid rods but the question that must be asked is: how stable?

In an early study of the length of TMV, Williams and Steere (1951) made the following generalisations: (1) the monomer is usually in greatest abundance (2) some particles considerably shorter are always found (3) any post-extraction treatment causes the distribution curve of the lengths to broaden, (4) even for the monomer a fairly broad distribution of lengths is reported, (5) the nett result of changes during preparation must always be to broaden the distribution of lengths.

Pirie (1957) considered there were two main sources of size variation - shearing forces due to maceration, and solution and drying forces. This latter factor was also later confirmed by Horne (1967a).

In a detailed study of the alkaline degradation of TMV (Harrington and Schachman, 1956) it was found that the particles were broken down in a specific manner, presumably due to special structural features. They also found that a percentage of 300 nm long rods persisted indefinitely under the conditions of the experiment and that the persistence was not due to the existence of an equilibrium between disintegration and formation. Some particles seemed simply to be resistant to breakdown. This has been later confirmed by Pelcher and Halasa (1979a) and they estimated the resistant proportion as 15-20%. They found that the progeny of this resistant group was again sensitive to alkaline degradation. They also found (Pelcher and Halasa, 1979b) that tomato isolates were more sensitive than tobacco isolates.

In reference to this breakdown, Knight (1975) thought that it may possibly be that the interaction between protein and nucleic acid was not uniform along the length of the rod, being stronger at one end than at the other.

Before examining the effects that IEM has on a population of Tobamoviruses, the above discussion, which has a bearing on this, should be summarised.

It seems as if the published figures for particle size of Tobamoviruses may in some instances have been the result of subjective judgement before publication. If both definite and tentative members are considered some Tobamoviruses are uniparticulate and others multi-particulate and that both SHMV and ORSV should be included in the latter group. The less-than 300 nm particles do have a function in that they can induce polypeptide synthesis in an <u>in vitro</u> translation system and one of these proteins is most probably the coat protein.

There is evidence that many physical and chemical factors can be implicated in the breakdown of virus particles, including plant proteases and drying forces during grid preparation. At least as far as degradation of particles by alkali is concerned the Tobamoviruses vary in sensitivity and the particles of a given strain vary in sensitivity, some (about 15%) being apparently resistant.

4.8.6. *The effects of IEM on Tobamoviruses.*

Taking cognizance of these factors, what happens when IEM is used to prepare particles of rod-shaped Tobamoviruses for particle size studies? There is no doubt that the percentage of particles observed less than 300 nm is greatly increased and therefore the population characteristics are changed. As a result of this great care should be exercised when attempting to use this method, especially on an unknown virus. When an unknown Tobamovirus is trapped the short particles cannot simply be ignored as it is not known whether the unknown virus is like potato mop-top (described as bimodal), ORSV (described as unimodal but has 2 peaks in the size distribution) or like ToMV (which is unimodal). Ignoring the short particles makes them all like ToMV.

This may at first seem to be in contradiction to statements made in a review of IEM (Milne and Luisoni, 1977) in which they claim they have not seen an increase in breakage using IEM preparations and that the normal lengths of TMV and a Potexvirus are unchanged by the procedure. Considering the appearance of preparations in my study, and the figures showing the increase in the proportion of small particles, it is extremely difficult to believe that such a change could not be seen. It may be, though, that by "not seen" they may mean that it did not show up in normal length calculations. As shown

earlier just describing a population by normal length is somewhat meaningless and does not give any idea of what is happening to a population and can result in a loss of useful comparative information.

4.8.7. *The origin of small particles present after IEM.*

When IEM is used to prepare a population of virus particles there seems to be two possibilities, that are not mutually exclusive, as to the origin of the small particles, and these are (1) that the particles are present in the natural population but are differentially attracted to the IgG coating on the grid (2) that the particles are a product of the preparative procedure.

The first of these explanations is unlikely as this would mean that, in the case of a virus such as ORSV with a very high proportion of small particles in a negatively stained sap preparation, there would be sufficient short particles present to be differentially attracted to the coated grid to the almost total exclusion of the 300 nm long particles. There is no evidence that this occurs, and on coated grids the proportion of small:large is approximately the same as in negatively stained sap.

I consider that the most likely explanation is that the short particles are a product of the preparative procedure. In fact this explanation could also be applicable to the short particles seen in negatively stained preparations of ORSV and SHMV, rather than the theory proposed by Beachy and Zaitlin (1977), or in addition to their theory of encapsidation of discrete and functional RNA molecules.

It seems that the particles of Tobamoviruses (and probably other groups also) could be markedly unimodal <u>in situ</u>. In the case of the Tobamoviruses this would mean the majority of particles would be ca. 300 nm long. Most biological populations show a variation in

resistance to external stimuli, both between populations and between individuals within a given population. There is no valid reason to suppose that this situation would be different for viruses, and it has already been shown in this discussion that such variation does occur in relation to alkaline degradation of Tobamoviruses. This variation has been shown to occur between viruses (Pelcher and Halasa, 1979b) and between individuals within a population (Harrington and Schachman, 1956; Pelcher and Halasa, 1979a).

It is not reasonable to postulate that there would be no such variability in resistance or susceptibility to physical forces exerted on the intact virus rods. I would suggest that this variability is one of the underlying explanations for the observed variations in particle size in Tobamovirus preparations, both when examined in negatively stained sap and also on grids coated with antiserum.

Thus if it is assumed that the natural, in situ, population of a Tobamovirus has the majority of particles ca. 300 nm long then the following could be hypothesised to explain some of the observed facts.

When the viruses are prepared for examination in the electron microscope the particles are exposed to plant proteases and then considerable physical forces due to drying on the grid. Evidence presented earlier in the discussion has shown that both of these are capable of disrupting virus particles including Tobamoviruses. Some virus populations (e.g. U1-TMV, ToMV, U2-TMV) only have a small proportion of particles susceptible to breakdown under these conditions, while others (e.g. SHMV and ORSV) have particles that are much more susceptible, and it may be that the breakage occurs near

the 3' end. This is certainly so with alkaline degradation. This would explain the occurrence of the S and I-2 particles in work previously described (Whitfeld and Higgins, 1976; Bruening et al., 1976; Beachy and Zaitlin, 1977).

During ordinary negative staining the viruses are free to move during drying. When IEM is used to prepare samples the virus particles are fixed to the grid film at a number of points and so the whole particle is not completely free to move during drying and this results in additional shearing forces at these fixed points. These forces are then sufficient to disrupt a considerable proportion of viruses such as U1-TMV, U2-TMV and ToMV which were resistant to normal drying forces. The observed populations of these viruses then appear similar to those of SHMV and ORSV prepared by negatively staining sap.

It may well be that viruses such as potato mop-top, that has been described as having particles of 100-150 nm and 250-300 nm, are in the same category as SHMV and ORSV, but happen to have been described by someone who did not decide to apply preconceived ideas of Tobamoviruses to the analysis of particle sizes. Potato mop-top has been examined by IEM (Roberts and Harrison, 1979) and it was found that the same two peaks were present but there was also an increase in smaller particles and several intermediate lengths.

4.8.8. *Concluding Remarks.*

The previous results and subsequent discussion have brought out and highlighted a number of points which will now be listed by way of summary, together with some brief comments on each.

(1) Whenever possible, especially in the case of elongated, rod-shaped plant viruses, particle sizing preparations should be

made by a technique using the minimum number of preparative steps. Usually this would mean negatively stained sap from infected leaves. The stain used should have a minimum effect in degrading particles, and so would militate against the use of PTA which is known to destroy many types of virus particles to varying extents. The more steps involved in a technique to prepare a virus sample for measurement, the more care should be taken in interpreting the results, and the further the results will be from the true, in situ, population of particles.

(2) When such a minimum preparation technique is used, the whole population should be taken into consideration preferably by histogram or ogive. Whether or not the small particles are functional or significant the presence or absence of them is diagnostic, and the sizes of them can be of value. As an example of this, the fact that there is a high percentage of small particles present differentiates SHMV and ORSV from other Tobamoviruses such as U1-TMV, U2-TMV, ToMV. The fact that the majority of small particles are ca. 40 nm in SHMV distinguishes it from ORSV in which the mean length of the smaller particles is ca. 100 nm.

(3) A single criterion such as normal length should not be used to describe the particle size of a population of any virus. A better criterion would be one that took account of all particles as by definition normal length only considers the main peak in a histogram. If the results cannot be presented graphically, or a mathematical description is needed for some reason, then an alternative to normal length should be found. I would suggest something like "modal means" whereby the mean of each peak in the distribution curve is given. However a graphic representation would at all times be preferable. The least useful description would seem to be the normal length.

(4) IEM is probably the most efficient way to obtain large numbers of virus particles on a grid to study particle morphology and, in the case of isometric virus particles, particle size. There is little doubt that the technique of IEM is capable of altering considerably the observed particle population of elongated, rod-shaped viruses. In such instances the greatest care must be taken in interpreting, analysing and reporting population sizes obtained by this means. In such cases the small particles cannot simply be ignored as they may be part of the population seen in negatively stained sap preparations (e.g. as in SHMV, ORSV, potato mop-top) or they may only be present due to the disruptive features of the method (e.g. as in U1-TMV, U2-TMV, ToMV).

(5) I consider that the increase in the number of small particles present after trapping is due to the added disruptive forces that occur as an intrinsic part of the method. It could well be that all Tobamovirus particles are ca. 300 nm in situ, that each virus varies in its tolerance to disruptive forces and that there is also a variation in tolerance between individual particles of one virus. Thus negatively staining expressed sap can be sufficient to break particles of SHMV, ORSV (and possibly viruses like Potato mop-top), whereas additional disruptive forces are necessary to break particles of viruses like U1-TMV, U2-TMV and ToMV. These additional forces are supplied by either purification or IEM preparation.

(6) While there is absolutely no doubt that, with many viruses, there is a great need to increase the number that are present on a grid, it is doubtful if IEM is the best way to do it in the case of rod-shaped viruses being prepared for particle measurement.

5. COMPARATIVE COUNTS OF TRAPPED VIRUS PARTICLES

"The guiding motto in life of every natural philosopher should be 'Seek simplicity and distrust it'."

A.N. Whitehead.

5.1. Introduction

Since the development of IEM by Derrick (1973b) there has been widespread use of the method for both detection and identification of plant virus particles, principally using crude plant sap extracts. Various factors can be involved in the sensitivity of the method for both purposes, and these factors cover most aspects of the technique.

In this section only the aspects of the technique involving the number of particles attached to a grid will be considered, and these factors are: antiserum diluents; the effect of antiserum dilutions and coating times; virus dilution and acquisition times; the role that precoating grids with protein-A may play.

There are many instances in which the number of particles trapped on a grid have been used diagnostically (Derrick, 1973b; Derrick and Brlansky, 1976; Paliwal, 1977; Milne and Lesemann, 1978; Lesemann et al., 1980; Roberts et al., 1980), and some of these report a limited amount of work on the effects of some of the above factors. The viruses used in these experiments cover a range of different groups including Tobamoviruses, Potyviruses, Luteoviruses and Reoviruses. There is no one report that covers experimentation on all of these factors using a single virus group as the experimental model.

This section aims to look at each of the factors mentioned above under similar conditions and using the same virus or virus group for comparative purposes.

5.2. Antiserum Dilution and Diluents

When a grid is coated with IgG, the reaction is not dependent on the concentration of the applied IgG until very high

dilutions are used. Until this point the grid will almost certainly have the maximum number of IgG molecules attached, and this should be particularly so with the longer coating times that are part of the more efficient IEM techniques.

On a grid that has not been pretreated with pA, and with a given concentration of virus in the sap to be tested, there would be little alteration in particle density over a considerable range of antiserum dilutions from ca. 1:1,000 and higher.

It is therefore somewhat unrealistic to use serum dilution end-points in a diagnostic manner based on particle density on the grids. While it may work for very distantly related viruses it would certainly not be practical for more closely related viruses.

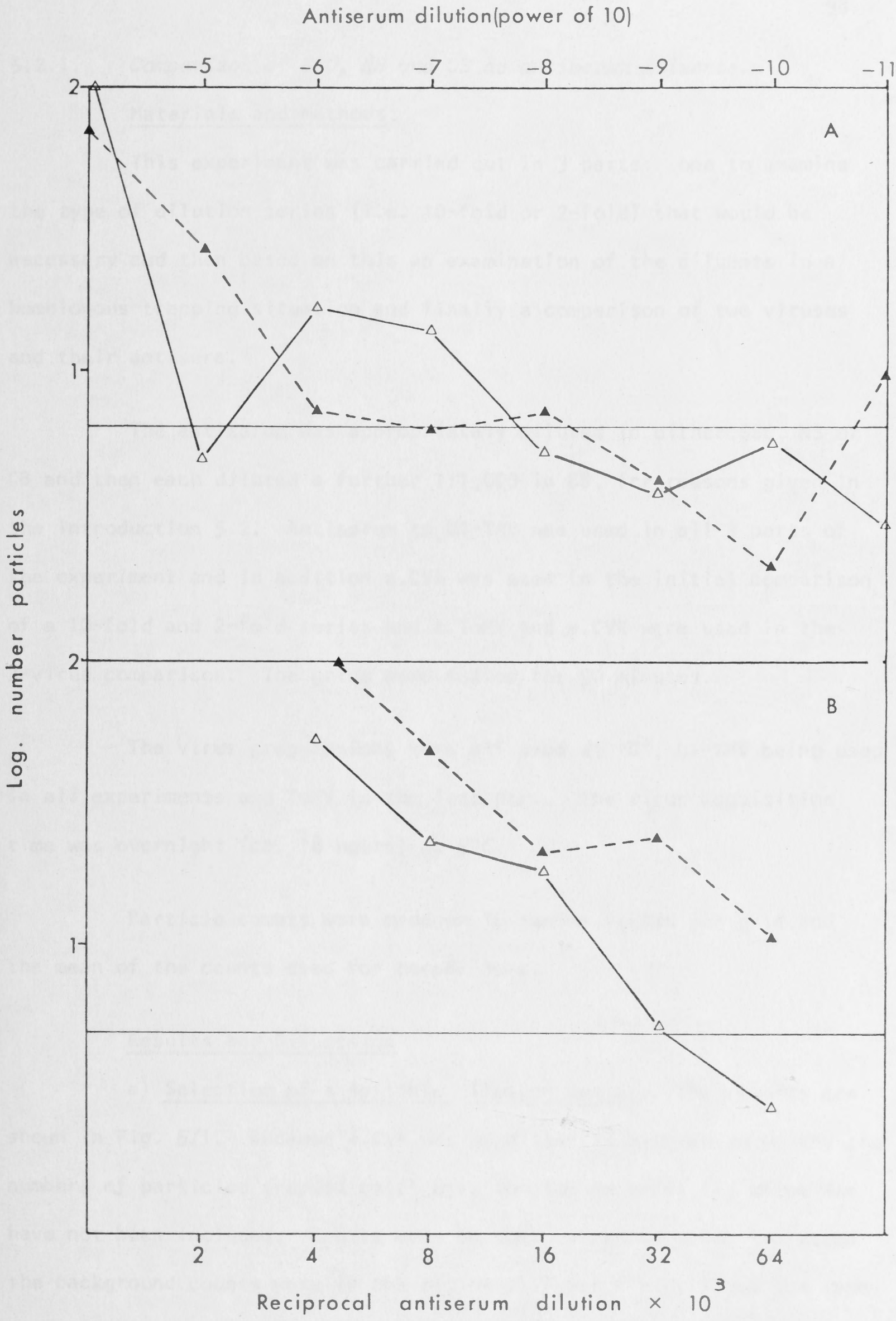
If the antiserum was diluted using a diluent that contained a competitor, such as IgG from normal serum (NS), then dilution end-points may be reached more rapidly and make such diagnostic tests more practical.

This set of experiments was therefore set up to examine the effects of dilution series of antisera using the usual diluent, CB, and also 2 others: SSC which is commonly used as an antiserum diluent and NS for the reasons stated previously.

In discussion 3.4 evidence was presented that there is a competitive inhibitor present in undiluted serum. With such an inhibitor present the dilution series would not give meaningful results as the reduction in particle numbers could be due to the presence of the IgG from the NS or due to the inhibitor. It is known that the effect of any inhibitor is greatly reduced after dilution of serum to 1:1,000 and so for this reason each of the dilutions in SSC or NS were then diluted 1:1,000 before use.

Fig. 5/1. The effect of using SSC (—— △ ——) and NS (- - -▲- - -) as diluents for a 10-fold (A) and 2-fold (B) dilution series of a.U1-TMV, on the number of particles of the homologous virus that were trapped. The horizontal line gives an indication of the background level of particles trapped on an uncoated grid or one coated with an unrelated antiserum.

Fig. 5/1.


Antiserum dilution(power of 10)
−5
−6
−7
−8
−9
−10
−11
2
1
A
B
Log. number particles
2
1
2
4
8
16
32
64
Reciprocal antiserum dilution × 10³

5.2.1. *Comparison of SSC, NS and CB as antiserum diluents.*

Materials and Methods

This experiment was carried out in 3 parts: one to examine the type of dilution series (i.e. 10-fold or 2-fold) that would be necessary and then based on this an examination of the diluents in a homologous trapping situation and finally a comparison of two viruses and their antisera.

The antiserum was appropriately diluted in either SSC, NS or CB and then each diluted a further 1:1,000 in CB, for reasons given in the introduction 5.2. Antiserum to U1-TMV was used in all 3 parts of the experiment and in addition a.CV4 was used in the initial comparison of a 10-fold and 2-fold series and a.ToMV and a.CV4 were used in the 2-virus comparison. The grids were coated for 90 minutes.

The virus preparations were all used at 10°, U1-TMV being used in all experiments and ToMV in the last part. The virus acquisition time was overnight (ca. 18 hours) at 5°C.

Particle counts were made on 10 random fields per grid and the mean of the counts used for comparisons.

Results and Discussion

a) Selection of a suitable dilution series. The results are shown in Fig. 5/1. Because a.CV4 was so distantly related to U1-TMV the numbers of particles trapped by it were too low to be of any value and have not been included. Counts made on some untrapped grids indicated the background counts were in the region of 5 per field, about the same as for the grids coated with a.CV4. At the higher dilutions the particles present were in such high numbers that they could not be counted and have not been included in the graphs.

Once the particle numbers were in a low enough density to be counted reasonably the 10-fold dilution series proved to be in too large steps and the numbers fell rapidly to background levels and so the 2-fold dilution series was chosen for the subsequent tests.

The 2-fold series showed that there was not much difference between the two diluents but that when NS was used it resulted in a slightly higher number of particles being trapped.

b) Trapping of homologous virus using 3 antiserum diluents. The effect of the 3 diluents are shown in Fig. 5/2. Up to a dilution of 1:8,000 there was very little difference between the diluents, with slightly lower numbers being present when carbonate buffer was used. However, once the dilution was greater than 1:8,000 the numbers trapped, when SSC or NS were the diluents, continued to fall rapidly whereas the numbers on the grids using CB as the diluent rose slightly.

When compared to the number of particles trapped at 1:1,000, at 1:64,000 there were only 2% of them trapped with SSC, 4% with NS and 64% with CB.

If the method is being used to detect relationships between viruses it is desirable to have the number of particles decrease continuously with antiserum dilution and from the experiments just described SSC or NS would seem to be better diluents.

There has been a previous report of NS being added to the trapping antiserum (Milne and Lesemann, 1978), and it was found that there was quite a large effect in decreasing the number of trapped particles. This apparent discrepancy can be explained in terms of the competitive inhibitor present in serum (see Discussion 3.4). They added the normal serum directly to the diluted antiserum. The NS

Fig. 5/2. A comparison of the effects of SSC (—— □ ——) NS (- - -■- - -) and CB (—— ○ ——) as antiserum diluents for coating grids. The effects on the number of trapped particles are shown using the virus U1-TMV and its antiserum.

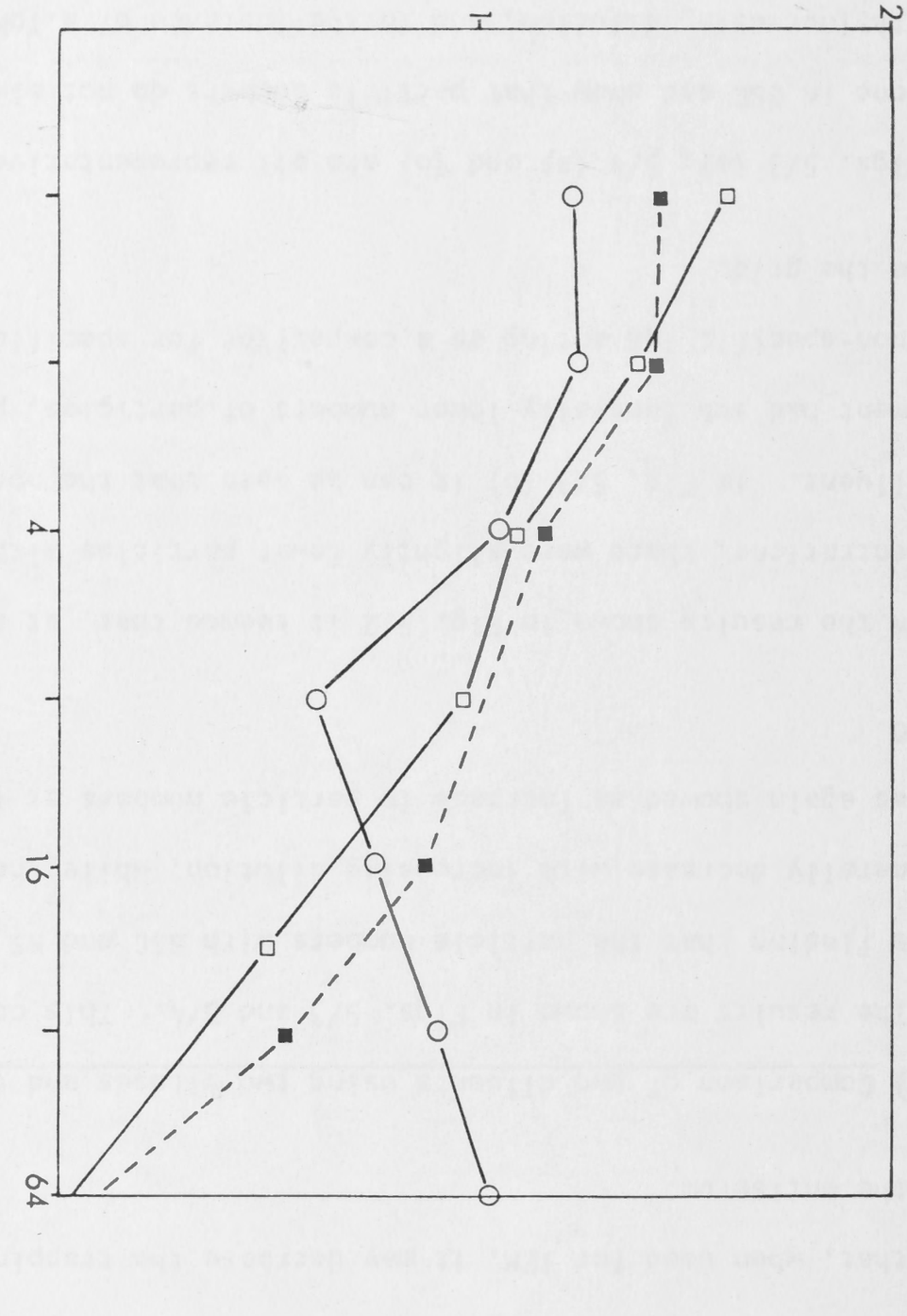

Log number particles
1
2
4
16
64
Reciprocal antiserum dilution $\times 10^3$

Fig. 5/2.

would therefore contain the inhibitors at a high concentration and the effect would be almost the same as using the specific antiserum at a higher concentration.

It is not known why SSC appears to be a poor diluent for this work as it is often used in serological tests as a diluent. However, it appears that, when used for IEM, it may decrease the trapping ability of the antiserum.

c) Comparison of two diluents using two viruses and their antisera. The results are shown in Figs. 5/3 and 5/4. This confirms the previous finding that the particle numbers with SSC and NS as diluents generally decrease with increasing dilution, while the carbonate buffer series again showed an increase in particle numbers at 1:16,000 and 1:64,000.

In the results shown in Fig. 5/2 it seemed that, at the higher concentrations, there were slightly fewer particles with the carbonate diluent. In Fig. 5/3 (b) it can be seen that the normal serum treatment had substantially lower numbers of particles, possibly due to the non-specific IgG acting as a competitor for specific IgG for sites on the grid.

Figs. 5/3 (a), 5/4 (a) and (b) are all representative of the dilutions done in SSC and show that particle numbers do not always decrease with increasing dilution, and in the instance of a.ToMV the numbers remain somewhat constant with dilution. Thus SSC cannot be relied on to produce a fall-off in particle numbers due to antiserum dilution.

No judgement can be made on the effectiveness of the diluents with a.CV4 as this antiserum was too distantly related serologically to

Fig. 5/3. The effect of antiserum dilution, and diluents, on the relative trapping ability of antiserum coated grids. These figures show the effect on numbers of particles of U1-TMV (A) when the 3 different antisera a.ToMV (—— ○ ——), a.U1-TMV (- - - ▲ - - -), and a.CV4 (—— △ ——) are diluted in SSC.

(B) When antiserum to U1-TMV is diluted in SSC (—— □ ——), NS (- - - ■ - - -) and CB (—— ○ ——).

Fig. 5/3.

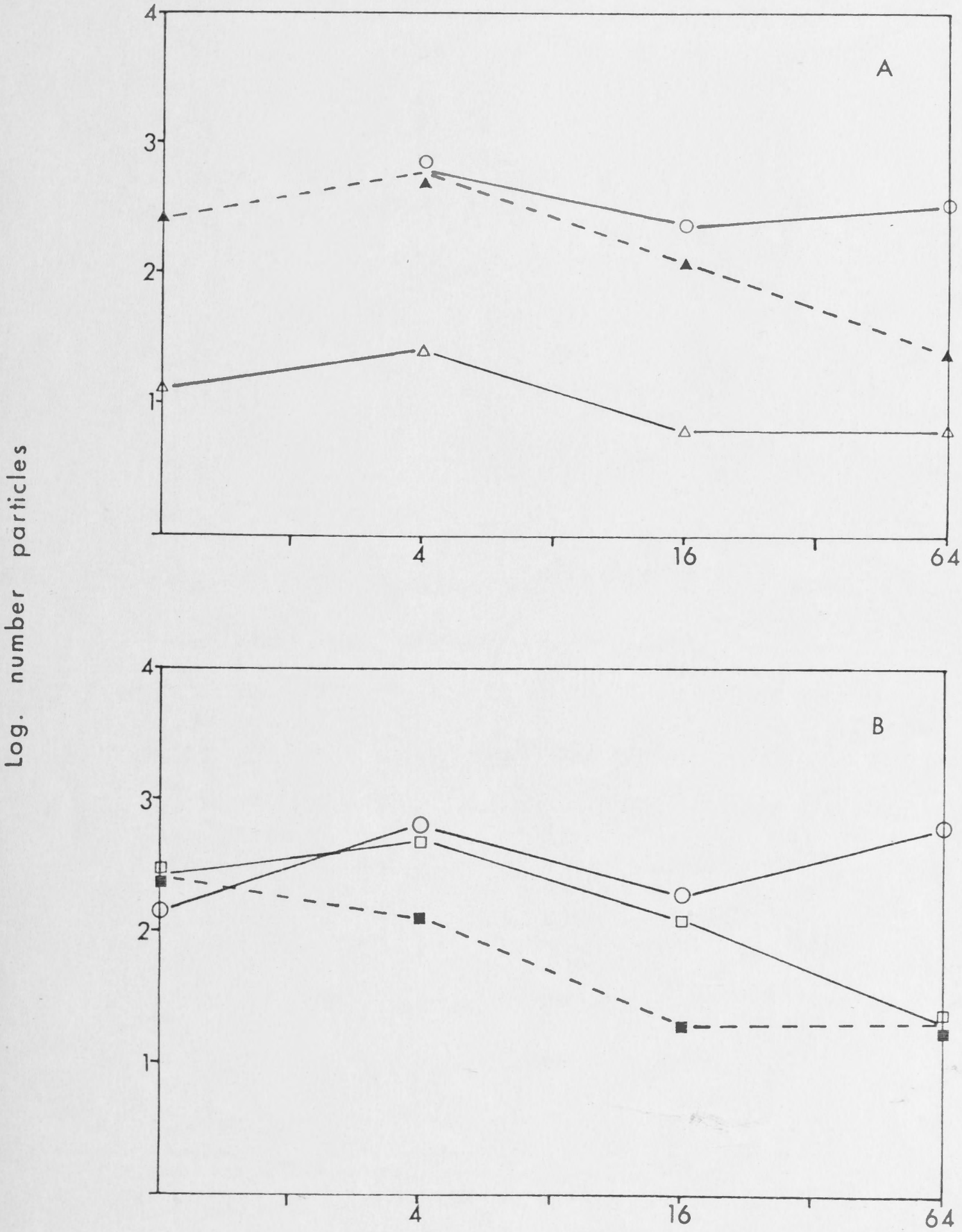
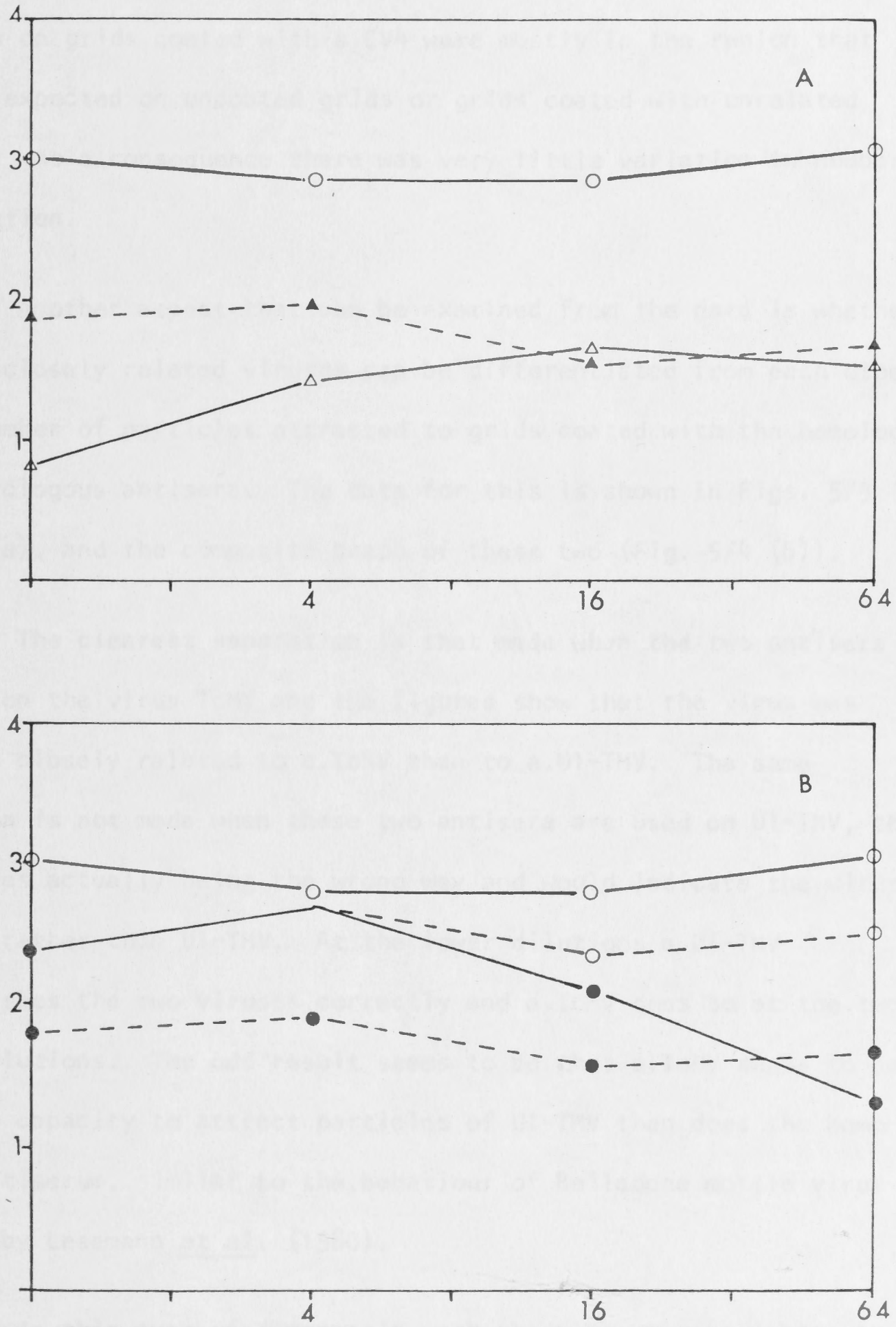

A
B
4
3
2
1
4
16
64
Log. number particles
Reciprocal antiserum dilution x10³

Fig. 5/4. Comparison of numbers of trapped particles on grids coated with homologous and heterologous antisera. (A) the effect of coating grids with antiserum to U1-TMV (- - - ▲ - - -), ToMV (—— ○ ——) and CV4-TMV (—— △ ——) on the number of particles of ToMV trapped. All the antisera were diluted in SSC.

(B) Both a.U1-TMV (●) and a.ToMV (○) were diluted in SSC and used to coat grids and the number of homologous (————————) and heterologous (- - - - - -) particles were counted.

Fig. 5/4.


A
B
4
3
2
1
4
16
64
Log. number particles
Reciprocal antiserum dilution × 10

the viruses used to pick up sufficient particles. The numbers of particles on grids coated with a.CV4 were mostly in the region that would be expected on uncoated grids or grids coated with unrelated antisera. As a consequence there was very little variation in numbers with dilution.

Another aspect that can be examined from the data is whether somewhat closely related viruses can be differentiated from each other by the number of particles attracted to grids coated with the homologous and heterologous antisera. The data for this is shown in Figs. 5/3 (a) and 5/4 (a), and the composite graph of these two (Fig. 5/4 (b)).

The clearest separation is that made when the two antisera are used on the virus ToMV and the figures show that the virus was much more closely related to a.ToMV than to a.U1-TMV. The same separation is not made when these two antisera are used on U1-TMV, the differences actually being the wrong way and would indicate the virus was ToMV rather than U1-TMV. At the lower dilutions a.U1-TMV distinguishes the rwo viruses correctly and a.ToMV does so at the two higher dilutions. The odd result seems to be that a.ToMV seems to have a greater capacity to attract particles of U1-TMV than does the homologous antiserum, similar to the behaviour of Belladona mottle virus reported by Lesemann et al. (1980).

In this type of diagnostic work there are two variables that cannot really be controlled and these are the density of IgG molecules on the grid surface and the concentration of virus particles in the plant sap preparation.

Considering that the coating of the grid with antiserum is not really concentration-dependent, and that the antisera to ToMV and

U1-TMV have similar titres, there should be approximately the same density of IgG molecules on grids coated with either.

The only variable that would most likely cause differences in counts would be that of virus particle concentration in the sap macerate. The general question of the type of effect of particle concentration will be taken up in section 5.5, but there is little doubt that the differences could be great, especially if the samples obtained were from plants at greatly different stages of infection. This factor would be of greater importance in field samples but in this experimental work any differences should not be too large as all plants were sampled when showing strong symptoms and had a high concentration of viruses in them. Variations would still occur, however, and this must be kept in mind when doing comparative studies on the number of particles present on a grid.

These results do indicate that differential particle counts may hold some promise as being a suitable method to obtain some diagnosis of viruses, particularly those that are not very closely related. To be of practical value, any differences should be easily detected rather than having to be statistically analysed. It seems that with two viruses such as ToMV and U1-TMV it may be possible to obtain such differences.

5.2.2. *Homologous and heterologous trapping using SSC at a higher final concentration.*

In section 5.2.1. the effect of diluents was examined by diluting the antiserum in one of the test diluents and then 1:1,000 in CB. This experiment was set up to see what effect would be obtained by having the SSC present in the final grid coating preparation at a higher concentration, i.e. by diluting the antiserum 1:1,000 in carbonate buffer and then doing the final dilution in SSC.

Materials and Methods

One half of the experiment was set up to compare the two viruses U1-TMV and ToMV and their antisera at three dilutions (1:4,000; 1:16,000; and 1:64,000). The dilutions were prepared by diluting the antisera 1:1,000 in CB and then an appropriate dilution in either SSC or CB.

The other half of the work involved 3 viruses (U1-TMV, U2-TMV and ToMV) and their antisera. The antisera were used at two dilutions, 1:1,000 in CB and this preparation diluted 1:16 in SSC.

In all instances the grids were coated for 90 minutes and then allowed to react with virus preparations overnight at 5°C.

Results and Discussion

The results are shown in Fig. 5/5 and Table 5/1. In the graph it can be seen that there was no pronounced decline in particle numbers with increasing dilution and that the virus ToMV was clearly identified by the two antisera, irrespective of the diluent. Likewise the diluent did not alter the fact that U1-TMV was not clearly identified by the two antisera.

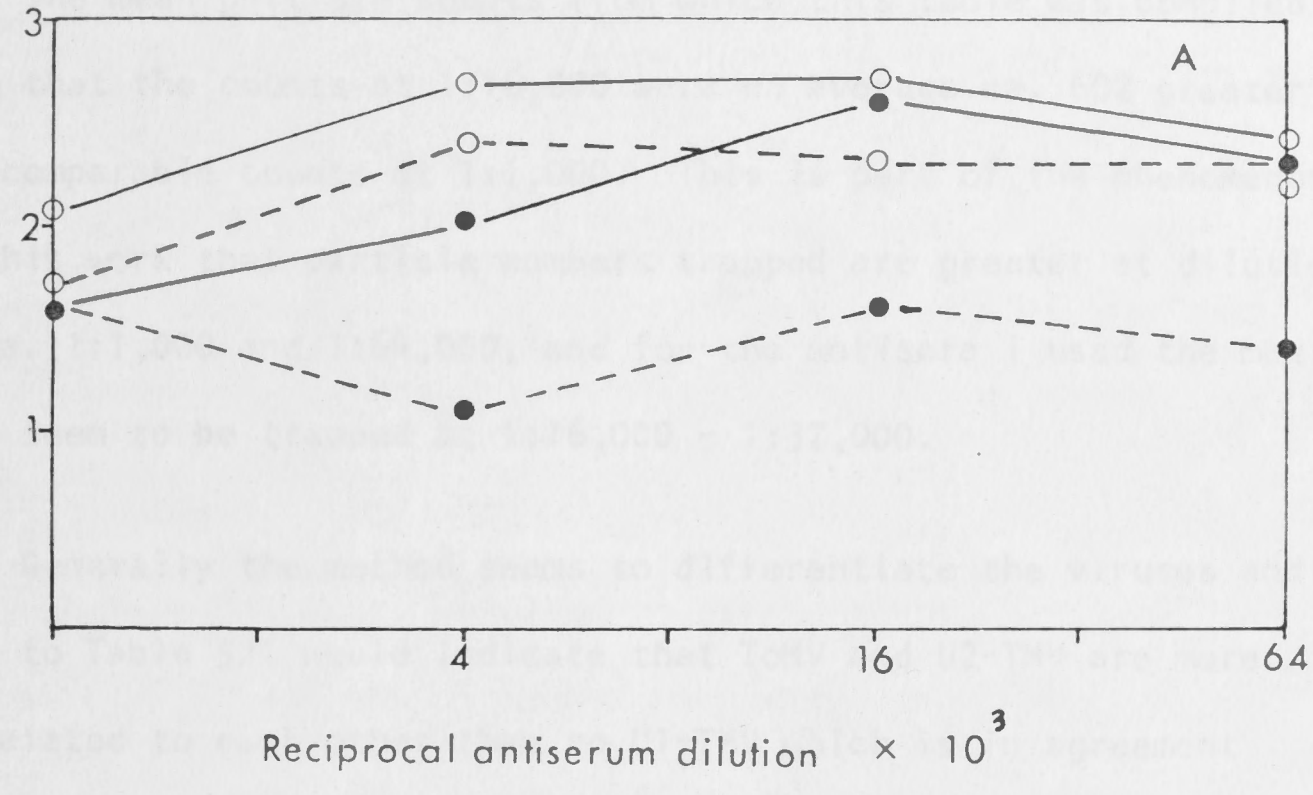
The two viruses were separated and diagnosed correctly when the counts on a single antiserum are counted. However, this method is impracticable as it is strongly influenced by relative virus concentrations.

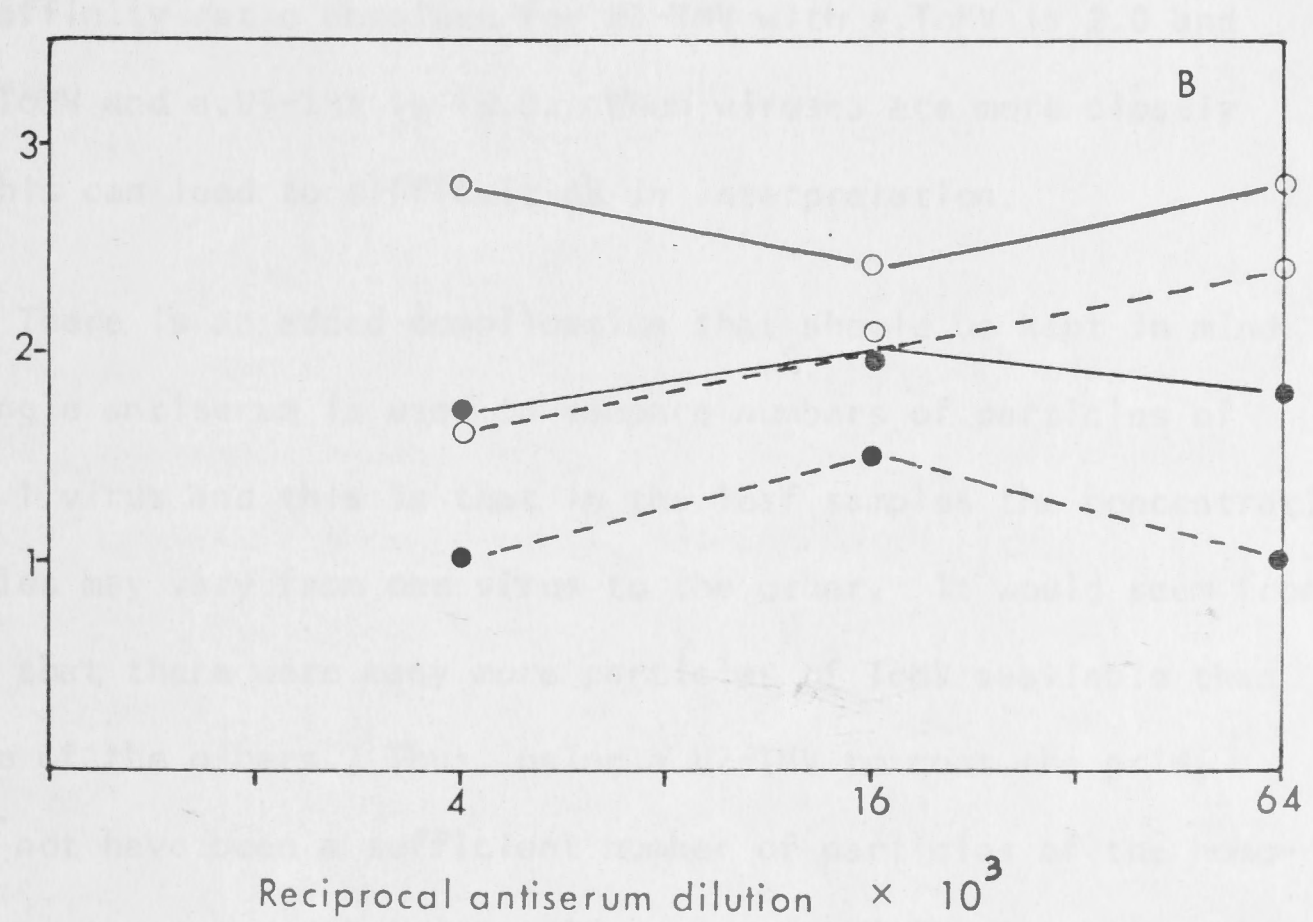
Table 5/1. Affinity ratios (see 2.3.3) based on virus particle counts using 2 antiserum diluents, carbonate buffer (1:1,000) and the same buffer followed by SSC (1:16,000).

VIRUS	ANTISERUM					
	1:1,000			1:16,000		
	a.U1-TMV	a.U2-TMV	a.ToMV	a.U1-TMV	a.U2-TMV	a.ToMV
U1-TMV	1.0	1.9	2.0	1.0	1.3	0.8
U2-TMV	8.7	1.0	-	6.1	1.0	3.1
ToMV	19.0	0.8	1.0	21.8	1.2	1.0

Fig. 5/5. Homologous (————) and heterologous (- - - - - - -) trapping with antisera to U1-TMV (●) and ToMV (○) using two diluents for the antisera, SSC (A) and CB (B).

Fig. 5/5.


A
3
2
1
4
16
64
Reciprocal antiserum dilution × 10³


B
Log. number virus particles
3
2
1
4
16
64
Reciprocal antiserum dilution × 10³

The mean particle counts from which this table was compiled were such that the counts at 1:16,000 were on average ca. 60% greater than the comparable counts at 1:1,000. This is part of the phenomenon seen in this work that particle numbers trapped are greater at dilutions between ca. 1:1,000 and 1:64,000, and for the antisera I used the most particles seem to be trapped at 1:16,000 - 1:32,000.

Generally the method seems to differentiate the viruses and reference to Table 5/1 would indicate that ToMV and U2-TMV are more closely related to each other than to U1-TMV which is in agreement with Gibbs (1977).

The table also shows the difficulties that can occur due to the fact that the heterologous titres of two viruses and their antisera can vary depending on which way the test is done. As an example of this the affinity ratio obtained for U1-TMV with a.ToMV is 2.0 and that for ToMV and a.U1-TMV is 19.0. When viruses are more closely related this can lead to difficulties in interpretation.

There is an added complication that should be kept in mind when a single antiserum is used to compare numbers of particles of more than 1 virus and this is that in the leaf samples the concentration of particles may vary from one virus to the other. It would seem from Table 5/1 that there were many more particles of ToMV available than there were of the others. Thus, using a.U2-TMV to coat the grid, there may not have been a sufficient number of particles of the homologous virus present so that it could trap more U2-TMV than it could ToMV. Because of this it would appear to be a better practice, with infected leaf tissues, to rely more on the relative particle numbers of a single virus trapped on grids coated with different antisera.

5.3. Coating and Virus Acquisition Times

As shown in section 3 there are a variety of modifications to the basic principle of the IEM method and two of the major variations are the antiserum coating times and virus acquisition times. The experiments in section 5.2 have shown that the method of comparative particle counting has some merit as a diagnostic method but that the results were sometimes equivocal. A variation in the application times of the reactants may help to maximise any differences in the relative particle numbers trapped by homologous and heterologous antisera.

It would seem that theoretically there could be a case for believing that the application times could play a role in maximising the differences between the particle numbers trapped by two antisera. In any antiserum the antiviral antibody molecules will all react with the homologous virus and some will cross-react with related viruses. The proportion of cross-reacting antibodies depends primarily on the degree of serological relationship that exists between the two antigens.

It is reasonable to assume that both the specific homologous IgG and the cross-reacting IgG would be present on a treated grid in the same ratio that was present in the original antiserum. That is, there is no reason to suppose that one type of IgG is basically different to another, in so far as they would have equal propensity to attach to a grid surface whether or not the grid has previously been treated with protein-A. It would also be expected that the ratio of cross-reactive antibodies would be the same regardless of the density of the antibody covering on a grid, i.e. whether a short or long antibody coating time was used.

It has been shown in section 3 that (a) the virus acquisition time is important in obtaining the maximum number of virus particles; (b) there is still an increase in particle numbers after an overnight (ca. 18 hour) treatment; and (c) the rate of increase in the number of trapped particles decreases with time due to the gradual fall in number of available attachment sites and a consequent decrease in the chance of contact between a virus particle and a free IgG molecule.

Because of these factors it would be expected that the truest indication of the relationship between viruses would be obtained when all possible attachment sites were filled, i.e., when saturation is reached. Such saturation conditions would be expected to be reached most easily by reducing the number of IgG molecules on the grid by a short coating time and increasing the chances of all sites being occupied by a long VAT.

One of the aims of this series of experiments is to look at these factors and determine what variation there is in relative particle numbers by examining the various combinations of short and long coating and virus acquisition times.

Another aspect of importance is whether the method of counting 10 spots per grid is sufficient or whether more grids should be used and, if so, whether the number of counts per grid could or should be decreased.

5.3.1. *The effect of short and long coating and virus acquisition times on virus identification.*

Materials and Methods

The two antisera a.U1-TMV and a.ToMV were used at a concentration of 1:1,000 and grids coated for either a short (10 minutes)

or long (180 minutes) period. Each of these treatments were then used to trap virus from a sap preparation of ToMV (diluted to 10^{-1}) onto 3 grids per treatment for either a short (10 minutes) or long (20 hours) acquisition time.

The 3 grids per treatment were used to determine whether 10 counts per grid was satisfactory or not and the following counting procedures were tested: (1) 10 counts on 1 grid (2) 10 counts on each of 3 grids (3) 5 counts on each of 2 grids (4) 3 counts on each of 3 grids. The means and standard errors of the means (SEM) for each set of counts for each treatment were calculated. For each counting method the SEM was calculated as a percent of the mean. The SEM's for counting methods (1) and (2) above were ca. 8% of the means. The value for methods (3) and (4) were ca. 13%, indicating that 10 counts on 1 grid gives as reliable a result as the other methods tested.

When the same experiment was repeated using a much lower virus concentration (10^{-4}) only 2 grids per sample were prepared and 10 counts were made on each and the means of the 20 counts taken for comparative purposes.

Results

While the SEM may have varied in relation to the mean, the actual means of the particle counts were very similar and the counting method did not affect the overall results. However, in the following table the figures given will be the means of the 30 counts in the experiment with the high virus concentration (10^{-1}) and the mean of 20 counts (10 on each of 2 grids) for the work with the low (10^{-4}) virus concentration. The data is presented in the form of affinity ratios and is shown in Table 5/2.

Table 5/2. The effect of short and long coating and virus acquisition times on the affinity ratios at two virus dilutions.

VIRUS DILUTION	COATING TIME/VIRUS ACQUISITION TIME			
	Short/Short	Long/Short	Long/Long	Short/Long
10^{-1}	12.6	8.5	6.9	6.6
10^{-4}	3.5	12.0	8.0	10.0

At the more dilute virus concentration the heterologous counts were very low and close, or equal to, background levels.

The figures show that the proportions of the numbers trapped by the two antisera are affected by the application times.

Because many of the counts with the 10^{-4} virus preparation were extremely low and thus give some false impressions, the figures for the 10^{-1} virus preparations will be discussed first.

There is little doubt that the major contributing application time in obtaining a large affinity ratio is the virus acquisition time and that a short VAT is desirable to obtain maximum differentiation between counts. It appears that the antiserum coating may have a very minimal role in altering the affinity ratio. As an example with a long VAT the value of the ratio is almost the same for both short and long antiserum coating. When a short VAT is considered the coating time does seem to affect the ratio.

These figures do not, at first, appear to agree with the hypothesis put forward in the introduction that the treatment to give proper distinction between two antisera would be the short/long. It is essential in this context to distinguish clearly between <u>maximum separation</u> of the values and the <u>true value</u> of the relationship of the two antisera to ToMV.

Retaining the assumptions made in the introductory remarks to section 5.3, a possible explanation can be offered for the results obtained.

With a short coating time there are fewer IgG molecules attached to the grid surface, and, in the case of the heterologous antigen, many fewer of the cross-reactive antibodies. The more distant the relationship of the heterologous antiserum, the rarer will be the cross-reactive antibodies, and hence the chance of contact of a virus particle with such an IgG molecule will be reduced. The shorter the virus acquisition time, the less chance there will be of suitable contact being made. Because the virus particles can react with any of the IgG molecules in the homologous antigen there is much more chance of suitable contacts being made. It would thus be expected that, under conditions of short/short application times, there would be a greater difference between homologous and heterologous counts. With a very distantly related antiserum it could be that there was insufficient time for any contact between a virus particle and a suitable cross-reactive IgG molecule. This would then tend to exaggerate any differences but would not indicate the true relationship that exists.

A similar argument would apply to the long/long treatment in that there could be too many antibodies of the homologous antiserum present to enable all of them to be occupied by virus particles. This would reduce the homologous count. Because there are only a low proportion of cross-reactive antibodies present in the heterologous antigen a long VAT may be sufficient to enable all of them to react with a virus particle. This would then mean that the heterologous count could be a true indication of cross-reactivity but the homologous count would be an underestimate, thus giving a low ratio of counts.

These expectations of a short/short and long/long treatment appear to be just what does happen. Under these conditions the truer indication would be that of the short/long ratio, in this instance ca. 7:1, indicating there was approximately 1 IgG molecule in a.U1-TMV that cross-reacted with ToMV for every 7 homologous IgG's in a.ToMV.

Many viruses do not occur in such high concentrations. Lowering the virus concentration should have the effect of decreasing the chances of suitable contacts being made between the antibody molecules and the virus particles. This effect should be more pronounced on the heterologous antiserum and it is conceivable that under some circumstances (e.g. the short/short treatment) that the heterologous count would not get above the low background counts that would be expected on an uncoated grid.

Because the heterologous count would be expected to fall more than the homologous the overall effect would be to increase the ratios. However, in the short/short treatment it could be expected that the homologous counts would also be low and approach background levels. This would have the effect of this treatment having a ratio that would approach 1.

Reference to Table 5/2 shows that these predictions are indeed what happened as all ratios, except that of the short/short treatment, were larger than before and the figure for the short/short treatment decreased markedly (i.e. approaching unity). In all cases the virus was identified correctly.

There was an indication in this work that the coating time had little influence in changing particle numbers and the major factors were the virus acquisition times and the virus concentration, two

factors that are closely inter-related. The effect of virus concentration will be examined in 5.5 and the coating and acquisition times will now be examined.

5.3.2. *The effect of coating and virus acquisition times on the trapping of virus particles.*

In experiment 5.3.1 an attempt was made to determine whether the efficiency of diagnosis could be improved by using various combinations of short and long reaction times. The results indicated that the coating time played a very minor role (if any) in the final particle numbers on either homologous or heterologous grids.

It is necessary to examine this apsect in more detail as described methods of IEM vary greatly in the coating times but there is no reasoning given for any of the selected times.

Materials and Methods

Antiserum against ToMV was diluted to 1:1,000 and used to coat grids for 10, 30, 60, 120 minutes. Each of these treatments was then used to trap virus from a preparation of 10^{-3} ToMV for 10, 30, 60, 120 minutes and 18 hours.

Because of an anomaly in the result for the 10/10 minute treatment, and to examine the effect of acquisition times in the region between 2 hours and 18 hours, the experiment was repeated. The coating times were 10 and 30 minutes and the VAT's were 10, 30 minutes, 2, 4, 6, 18, 24 hours.

Counts were made on 20 random fields per grid and the means of these counts used for comparisons.

Results and Discussion

The results are shown in Table 5/3 and Fig. 5/6.

Fig. 5/6. The number of particles trapped from a 10^{-3} crude sap preparation of ToMV is shown in relation to the virus acquisition times. There are two time ranges shown: from 10-120 minutes and 18 hours (bottom scale, solid line) and from 2 hours to 24 hours (top scale, dotted line).

Fig. 5/6.

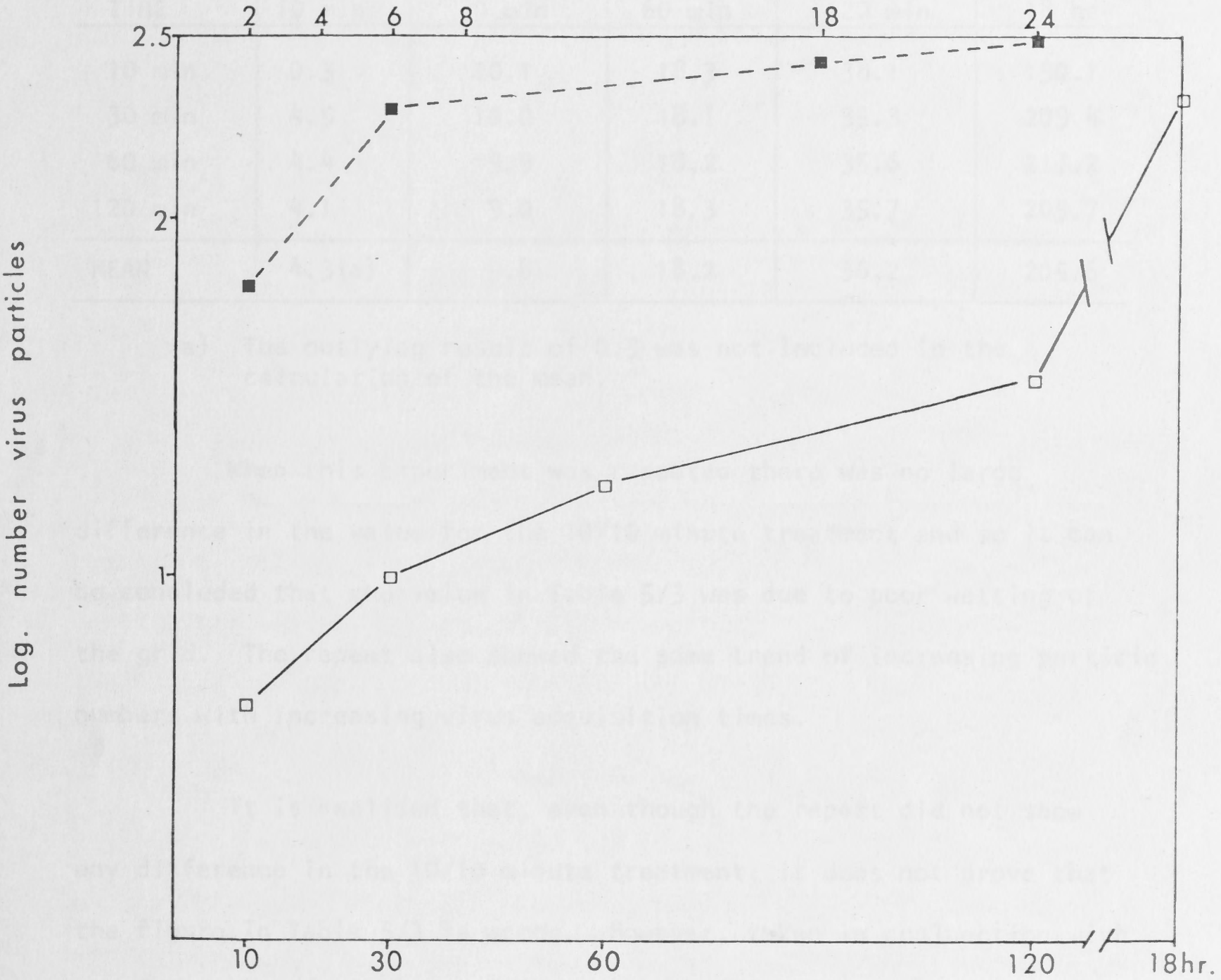

2
4
6
8
18
24
2.5
2
1
10
30
60
120
18hr.
Log. number virus particles
Virus acquisition time

Table 5/3. The effect of coating and virus acquisition times on the number of particles trapped on a grid. The figures are the means of 20 counts per treatment.

COATING TIME	VIRUS ACQUISITION TIME				
	10 min	30 min	60 min	120 min	18 hr
10 min	0.3	10.1	18.3	38.1	190.1
30 min	4.5	10.0	18.1	35.3	209.4
60 min	4.4	9.9	18.2	35.6	217.2
120 min	4.1	9.0	18.3	35.7	205.7
MEAN	4.3(a)	9.8	18.2	36.2	205.6

(a) The outlying result of 0.3 was not included in the calculation of the mean.

When this experiment was repeated there was no large difference in the value for the 10/10 minute treatment and so it can be concluded that the value in Table 5/3 was due to poor wetting of the grid. The repeat also showed the same trend of increasing particle numbers with increasing virus acquisition times.

It is realised that, even though the repeat did not show any difference in the 10/10 minute treatment, it does not prove that the figure in Table 5/3 is wrong. However, taken in conjunction with the facts that (a) the rapid IEM techniques utilise very short coating and acquisition times without any adverse effect and (b) I have not seen any such irregularities when testing the short methods (section 3), it would seem most likely that the figure in Table 5/3 is anomalous and may be ignored.

The results clearly show that the amount of virus present on the grid is dependent on the VAT and not on the antiserum coating time. Under the conditions of this experiment the coating time had no effect on particle numbers whereas the VAT resulted in large increases in particle numbers. Increasing the VAT from 10 minutes

to overnight resulted in a 48-fold increase in particle numbers.

While long acquisition times have little effect when higher concentrations of virus are present, there is a steady increase in numbers up to at least 24 hours when the virus concentration is low. This is very important from the point of view of virus detection. Figure 5/6 shows the changes in rates of increase in numbers. There is a large increase between 2 and 18 hours but when the intermediate times are tested it can be seen that a large portion of the increase is between 2 and 6 hours.

Even though the rate of increase in particle numbers was low by 18 hour reaction time it may have been due to the fact that, because of the low virus concentration, the incidence of contact between virus particles and attachment sites was low. This could mean that there were still a lot of sites left for virus attachment.

This was tested by using an overnight acquisition time and a range of virus preparations from 10° to 10^{-3} (which was used in these experiments), and comparing the numbers of particles attached to the grids. When this was done there was approximately the same number of particles attached as shown in Table 5/3 for 18 hours. Fig. 5/7 shows that as the virus concentration increased so did the particle numbers up to 10^{-1} and this was in the order of a 3-fold increase over the numbers at 10^{-3}. It would thus seem that with the lower concentrations there would most probably be a continuing slow increase in the number of particles trapped if the VAT was increased to times much greater than 24 hours.

It was also possible that, even though the coating time did not have any effect on particle numbers at the virus concentration used, some increase may be seen under very limiting conditions of a

Results

The affinity ratios are shown in Table 5/6, and it should be noted that the results are in two parts, the virus preparation for the dilutions 1:20-1:2,000 being different to that for the dilutions 1:4,000-1:16,000. The ratios in Table 5/6 have been calculated in two ways: (a) The first two lines of the table compare the numbers of particles of one virus trapped by each of the two antisera and (b) the second two lines compare the numbers of particles of each of the viruses trapped by a particular antiserum. The results are also shown in Fig. 5/8.

Table 5/6. Affinity ratios (p. 44) of virus particles trapped on antiserum-coated grids pre-treated with protein-A. The figures in the top half are calculated from the counts of particles trapped by the two antisera and those in the bottom half from counts of the two viruses trapped by each antiserum.

VIRUS	ANTISERUM DILUTION					
	1:20	1:200	1:2,000	1:4,000	1:8,000	1:16,000
U1-TMV	1.1	1.2	0.6	2.6	1.3*	1.5*
ToMV	3.2	11.8	12.6	4.0	5.2	2.7*
ANTISERUM						
a.U1-TMV	5.1	7.4	6.6	3.5	2.4*	1.4*
a.ToMV	0.7	1.9	1.0	2.9	2.7	2.8*

*Both counts in background region.

The results of the third experiment to examine the change in particle numbers with antiserum dilution are shown in Figure 5/9. The number of particles trapped decreases by 86% when the antiserum is diluted from 1:20 to 1:16,000.

Discussion

The particle numbers used to calculate the ratios indicated that there was a steady decline as the antiserum was diluted from 1:20 to 1:16,000. As this part of the work was done in two parts there could be no certainty on this point. However the subsequent

Experiment 5.4.1 was set up to use these two facts to determine whether a clearer identification of the virus would be obtained by using grids treated with protein-A and dilute antiserum.

5.4.1. *The use of protein-A and the effect of antiserum dilutions.*

Materials and Methods

The experimental procedure was carried out in 3 parts, the first two using different ranges of antiserum dilutions to look at the affinity ratios of particle numbers and the third to examine the use of a dilution series on particle numbers of a homologous virus-antiserum combination.

The first two experiments used the two viruses U1-TMV and ToMV at a concentration of 10°, and their antisera. The virus acquisition time was 30 minutes in both of these experiments. The antiserum dilutions, in CB in the two experiments were (a) 1:20, 1:200, 1:2,000; (b) 1:4,000, 1:8,000, 1:16,000.

Grids treated with protein-A for 10 minutes were coated with the antisera for 10 minutes before being put onto the virus preparations.

In the third experiment protein-A treated grids were then coated with a.ToMV for 10 minutes, the antiserum being used at all of the above dilutions. These grids were then used to pick-up virus particles from a 10^{-1} sap preparation of ToMV for 30 minutes.

Counts on all grids were made on 10 random fields and the means of the 10 counts then used to calculate the affinity ratios in the first two experiments and to examine the effect of antiserum dilutions on the number of trapped particles in the third experiment.

gives a greater separation of the homologous and the heterologous counts but the truest indication of the level of the relationship is most likely to be obtained with longer acquisition times.

Particle counting can be used to determine relationships between viruses or to obtain an identification but care must be exercised in the interpretation of the results. Due consideration must be taken of the times used, the virus concentrations and the closeness of serological relationships between the viruses from which the test antisera were prepared.

5.4. The effect of precoating grids with protein-A

The ability to use IEM for complete or partial diagnosis of a virus by use of different antiserum coatings on a grid is invaluable. I have shown that this can be done by utilising comparative particle counts for the antisera and that in order to get maximum differentiation it is desirable to use a short virus acquisition time. This means that a rapid method is better than a long one providing the virus occurs at a reasonable concentration to enable the particle counts to be greater than expected background levels.

In section 5.3 it was proposed that decreasing the number of available IgG molecules on a grid would maximise the diagnostic use of particle counts. It was established that this could not be done by decreasing the antiserum coating time to 10 minutes. It was shown in section 3 that when grids were treated with pA, and dilute antiserum used to coat them, there were fewer available sites for virus particles to attach to IgG than on grids not pretreated with protein-A.

This information can now be used to see what would be expected when a virus was trapped by two antisera using a short and a long VAT.

Because there are fewer sites on the grid coated with the heterologous antiserum, the reduction of the reaction time with a virus would have a greater effect on the number of particles and so the affinity ratio would be greater than with a long reaction time. This would give a better separation of the two antisera but a longer acquisition time would probably give a truer indication of the relationships between the two. This is in fact what is shown in the results in section 5.3.1.

The situation in 5.3.2 was further complicated and this makes predictions much more difficult. The basic reason for this is that the virus was present at a very low concentration and so the particle numbers were very low, many hardly above expected background levels. Under such conditions the amount of virus on the grid coated with heterologous antiserum could be very variable and this is shown by the fact that one of the ratios with a short virus reaction time was very high and one was the lowest.

If the virus concentration were sufficient to give heterologous counts slightly above the background level it would be expected that the affinity ratio for the short acquisition would be high and the long acquisition times would have lower ratios.

This set of experiments has established that the antiserum coating time has no role in determining the particle numbers above 10 minutes, and that the key factor is the virus acquisition time which is closely related to the virus concentration in the preparation. With higher virus concentrations a short reaction time with the virus

and Luisoni (1977) the virus preparations were such that the viruses were present at relatively high concentrations and hence only a much shorter time was needed to occupy a high proportion of sites and the rate of increase in virus particles with time then decreases markedly. In my work the virus was used at a very low concentration and the rate of increase in particle numbers was still quite substantial even after 18 hours.

This inter-dependence of virus concentration and acquisition time is also confirmed in some work reported by Roberts and Harrison (1979). In this report they examined potato leaf-roll virus (PLRV), that occurs in very low concentrations, and potato mop-top virus (PMTV) that occurs in much higher concentrations. With PLRV the particle density increased rapidly up to 4 hours and then more slowly up to 20 hours. With some samples of the same virus that occurred at higher concentrations there was little increase after 4 hours. However, with PMTV, attachment of particles was rapid and decreasing the reaction time from 4 hours to 45 minutes only halved the number of particles.

c) <u>The effect of treatment times on the ratio of particle numbers trapped by homologous and heterologous antisera</u>.

The arguments and assumptions used to predict the effect of treatment times was based on the fact that both the coating and virus reaction times played a part in the determination of the number of particles. Subsequent experimentation on the ratios indicated that the virus acquisition time determined the outcome and the coating time had little (if any) effect. Further examination of the effects of each of these treatment times showed that this was true and that the IgG coating of a grid occurred within the first 10 minutes.

was chosen. The times used by various workers vary from 5 minutes (Milne and Luisoni, 1977; Milne and Lesemann, 1978; Lesemann et al., 1980) to 90 minutes (Plumb, pers. comm.).

It would seem that such variations are unnecessary, at least for times greater than 10 minutes. I have not tested the effect of coating times less than 10 minutes as a reduction to 5 minutes does not have much practical value, because when many samples are being examined at the same time the following steps of washing a lot of grids and putting them onto a virus suspension usually requires 10 minutes or longer.

My results establish for the first time the relative values of antiserum coating and virus acquisition times in increasing the sensitivity of IEM for detecting virus particles. It is evident that coating a grid with serum for longer than 10 minutes has no effect and the key factor is the virus acquisition time.

In contrast to this it has been reported that an increase in the acquisition time from 1-16 hours only results in a 5-fold increase in the number of virus particles (Derrick, 1973b) and that 60 minutes gave little improvement over 15 minutes (Milne and Luisoni, 1977). In my experiments the increases from 1-16 hours were of the order of 11-18-fold and the change from 15-60 minutes was of the order of 3-fold.

These reports are not necessarily inconsistent as the two factors of virus concentration and reaction time needed to obtain occupation of available attachment sites are highly inter-dependent. The higher the virus concentration the less time is needed to occupy a high proportion of sites. In the work of Derrick (1973b) and Milne

As an example of the variation in methods the following 3 examples are cited. In one of the early papers describing the IEM method, Derrick (1973b) stated that he took a series of micrographs and counted from projections of the negative onto a screen. No mention is made as to whether the micrographs were from the same or different grid squares.

To evaluate the effectiveness of protein-A in IEM, Shukla and Gough (1979) counted from 12 micrographs taken randomly from 3 grid squares and then took a mean of these 12 counts.

Lesemann et al., (1980) studied some Tymoviruses by IEM and to compare different treatments they counted the particles in 10 binocular fields, each in a different grid square on each of 2 grids. For comparison they used the mean of these 20 counts. They found that repeated counts of groups of 10 from one grid, under their conditions, had a variation of ca. 15%. They do not give the errors present in the system that they actually used.

All these methods appear to work equally as well as each other. Under the conditions of my experiments it did not appear to matter whether the 10 counts were all taken from 1, 2 or 3 grids or whether a total of 30 counts (10 from each of 3 grids) was used. The overall findings were the same.

b) The effect of antiserum coating and virus acquisition times.

There has been a limited amount published on the effect of antiserum concentrations and virus acquisition times but there seems to be no published reports on the effect of the times used to coat the grids with antiserum and each modification of the method has a specific time without any explanation as to why the particular time

very low virus concentration. This was examined using a 10^{-5} virus preparation and coating times of 10, 30, 60 and 120 minutes, but there was no change in the number of trapped particles.

5.3.3. *Discussion.*

a) Method of obtaining comparative particle counts.

There have been no detailed studies on what might be the best method for obtaining a mean value for a particular treatment. One of the most detailed methods is that of Roberts (1980).

Basically the method relates to the conversion of the counts to the number of particles present in a "standard area" and takes into account many factors including such things as the relative tilt of the screen to the electron beam. I would consider that such a detailed method has its main application in a situation in which it was necessary to compare counts directly that have been made in different laboratories. Such an occurrence would be extremely rare and of doubtful value because of a number of variables that would occur in different laboratories. These variables, which could not be readily evaluated, would include variations in antisera and variations in virus concentration in the sample. The latter would be particularly noticeable where infected leaf macerate was used as the virus source, as most commonly occurs with IEM.

For the most part the particle counting method would be expected to vary from one laboratory to another, but the important factor should be that the counts reflect any differences that may be present in a given experiment without too large an error. Even if no mention of error is made published results can reflect the differences in the experimental system.

Fig. 5/7.

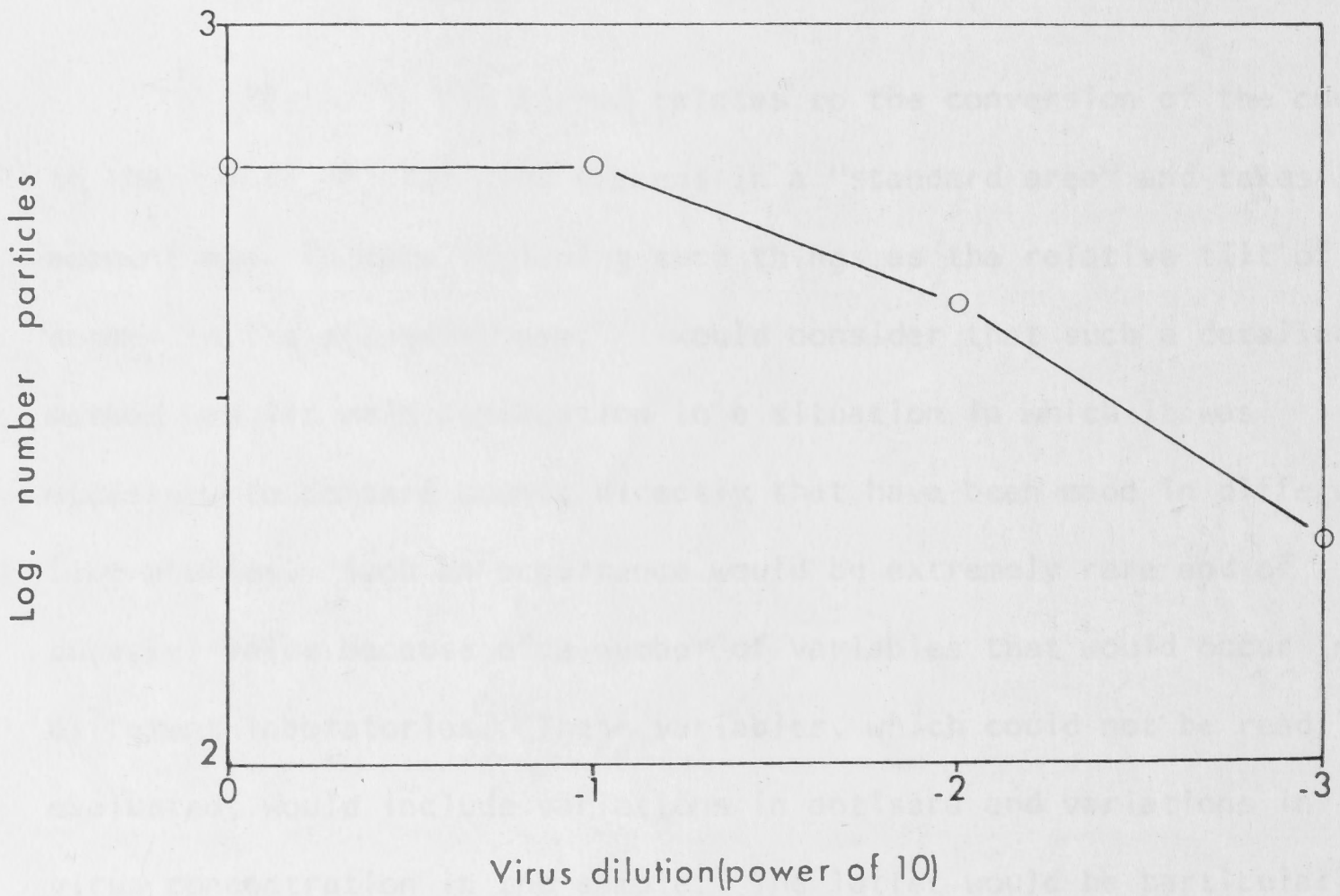


Fig. 5/7. The effect of virus dilution on the number of particles trapped using the virus ToMV and its antiserum.

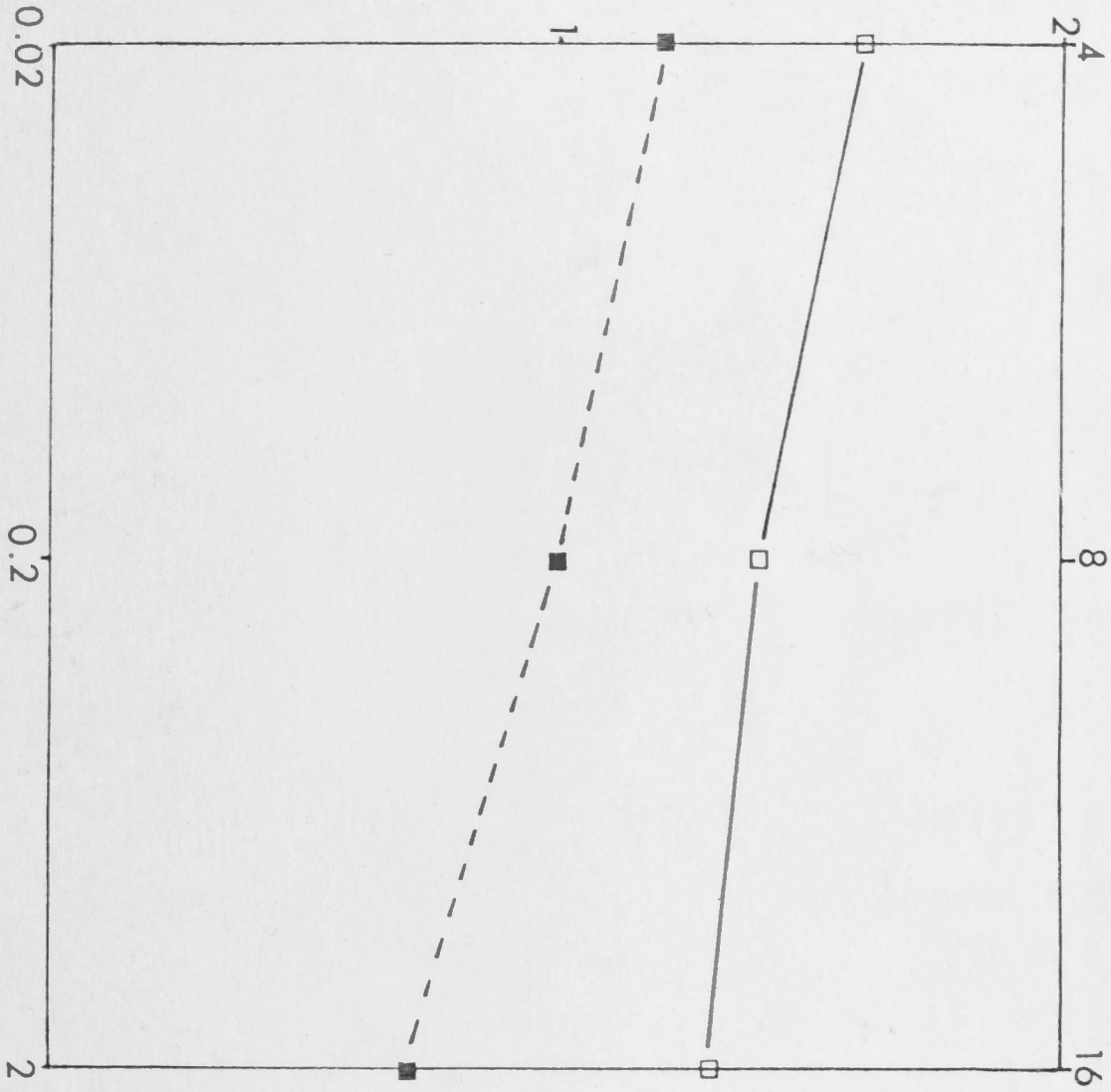

Log number particles
0.02
0.2
2
1
2
4
8
16
Reciprocal antiserum dilution x 10^3

Fig. 5/9.

Fig. 5/9. The effect of protein-A on a grid before antiserum coating. The figure shows how the number of particles of ToMV are affected by dilution of the homologous antiserum in two separate dilution series 1:20-1:2,000 (bottom scale, ————) and 1:4,000-1:16,000 (top scale, - - - - - -).

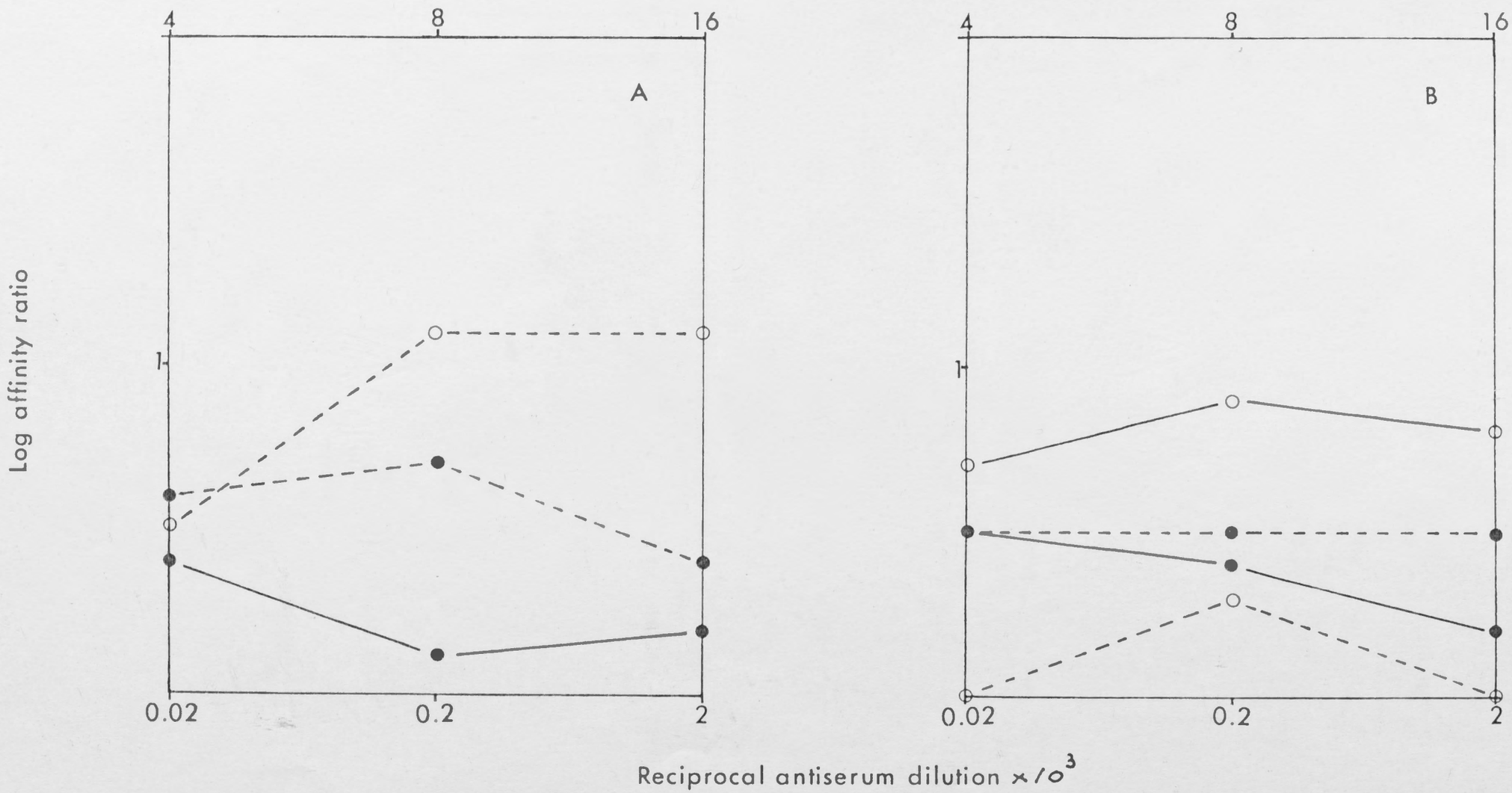

4
8
16
A
B
1
Log affinity ratio
0.02
0.2
2
Reciprocal antiserum dilution $\times 10^3$

Fig. 5/8.

Fig. 5/8. These graphs show the effect of antiserum dilution in 2 dilution ranges, 1:20-1:2,000 (○) and 1:4,000-1:16,000 (●) on the differentiation of the two viruses and the antisera to them, U1-TMV (solid line) and ToMV (dotted line). The measure of differentiation is the log of the affinity ratio. (A) shows the separation given by one virus with the two antisera and (B) shows the antisera, each comparing the counts of the two viruses. The grids were coated with protein-A before use.

check showed that this decline does occur.

However this only seemed to occur when the two antisera were compared using ToMV where the ratio increased between the dilutions of 1:20 and 1:2,000 and then the number of homologous attachment sites started to decrease and the ratio decreased and was approaching unity by a dilution of 1:16,000 when particle numbers were very low.

When an antiserum concentration of 1:20 was used there were high numbers of IgG molecules present on the grids and with a high virus concentration, such as that used, there was a reasonable chance that a high percentage of available sites were filled on both homologous and heterologous grids. As seen in the previous section this would lead to a poorer separation but a truer indication of the relatedness. This seems to be what happens. As the antiserum is diluted to 1:200 and 1:2,000 the number of sites decreases but is still within reasonable limits to trap sufficient virus particles. However the reduction in sites leads to a greater decrease in heterologous numbers and the ratio increases to give better separation. After the dilution becomes greater than 1:4,000 the number of sites becomes too small to trap many particles, at least with a 30 minute reaction time, and the numbers both tend to be close to background and the ratio gets smaller. These very small ratios, due to low particle numbers, bear no relation to the serological closeness of the virus, but rather show the poor trapping ability of the low IgG density and the 30 minute virus acquisition time.

It therefore does not seem that pre-coating the grids with pA is of any particular value and it appears to be less reliable than the previous method that did not use protein-A.

5.5. The effect of virus dilution on particle numbers and the affinity ratio

The preceding experiments described under sections 5.2 to 5.4 have shown the importance of the time the antiserum-coated grid reacts with the virus suspension. It has also been shown that altering the virus concentration from high to low can affect the number of particles trapped on a grid and the affinity ratio. In fact the virus concentration and virus acquisition time are intrinsically related, and have the same outcome on the final numbers of virus particles trapped on a grid.

It would be expected, therefore, that a decrease in virus concentration would have a similar effect to a reduction in virus acquisition time, i.e. a reduction in the number of trapped particles and an increase in the differential between homologous and heterologous counts as the virus concentration decreases.

The concentration of virus particles in sap extracts can range from very high (e.g. Tobamoviruses) to very low (e.g. Luteoviruses, Cryptic viruses, and any virus in certain stages of the infection process). Because of this it is important to examine the relationship that exists between particle numbers on the grid, the comparison of homologous and heterologous counts and the concentration of virus in the original preparation. The experimental examination of these factors was done using (a) purified virus and (b) infected sap.

5.5.1. *Purified virus: the effect of virus concentration on particle numbers trapped on grids.*

Materials and Methods

Using a purified preparation of virus often means that at the higher concentrations there is so much virus trapped on the grid that counting becomes impossible. In order to avoid this situation,

if possible, the antiserum a.ToMV was diluted to 1:100 in CB instead of the usual 1:1,000. The grids were coated with this for 30 minutes.

The virus concentrations that were used were

(a) a ten-fold series from 10° - 10^{-11}

(b) a four-fold series from 1:4 - 1:1,024.

A different purified preparation of ToMV was used for each and consisted of a second high speed pellet from a differential centrifugation. In both cases the pellet was re-suspended in 1.5 ml PB. For the ten-fold series this was diluted 1:20 and this was called 10°. For the four-fold series the resuspended pellet was the starting point.

Coated grids were treated with each of these dilutions of virus for 90 minutes and counts made on 20 fields per grid, 2 grids per treatment. The means of these 40 counts were used for comparative purposes.

Results

The counts are shown in Fig. 5/10, and will be discussed in conjunction with those from the experiments on sap extracts.

5.5.2. *Sap extract: The effect of virus concentration on particle numbers trapped on grids.*

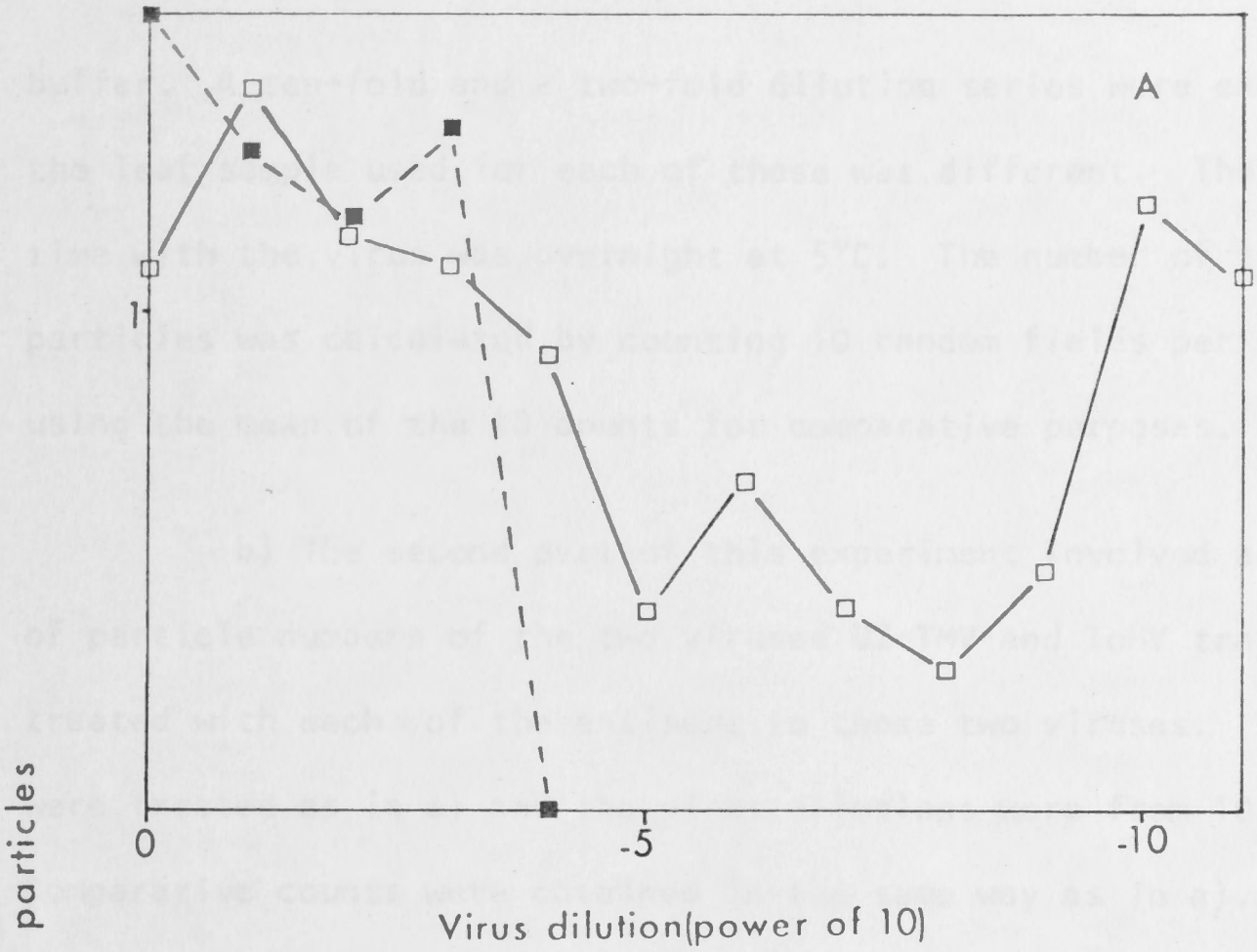
Materials and Methods

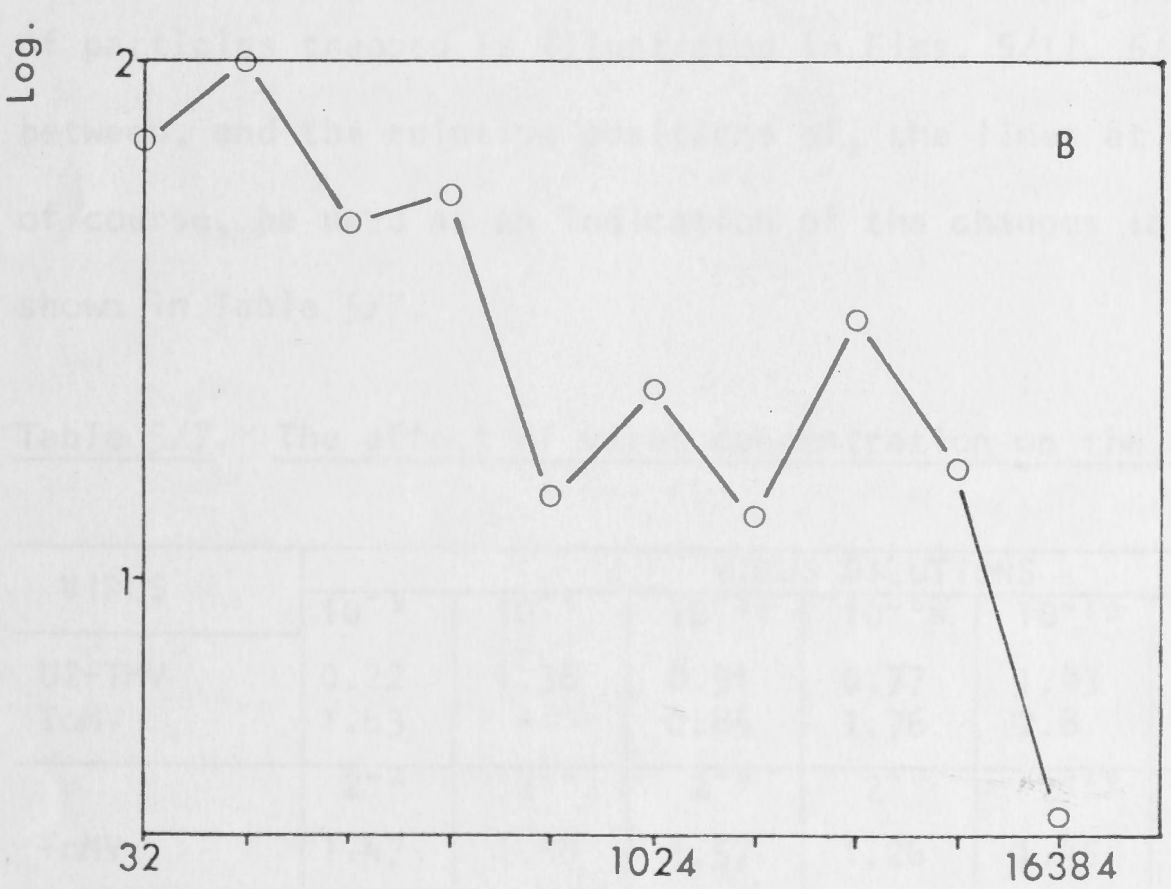
a) The first part of this experiment was designed to examine the effect of virus dilution on the number of particles of a single virus trapped onto grids coated with homologous and heterologous anti-sera.

The grids were coated with a.U2-TMV and a.ToMV diluted 1:1,000 in CB and the coating time was 60 minutes. Two virus dilution series were examined, the starting point for each being tomato leaf tissue, infected with ToMV, being ground in PB at the rate of 1 g leaf per 3 ml

Fig. 5/10. The effect of virus dilution on the number of particles trapped on an antiserum-coated grid.

A purified suspension of ToMV was used as the basis for a 10-fold (A) and a 2-fold (B) dilution series and these preparations were reacted for 90 minutes with grids coated with homologous antiserum. The effect of varying the virus concentration on the number of particles trapped is shown. Trapped preparations: ——— ; untrapped: - - - - - - .


A
1
0
-5
-10
Log. number particles
Virus dilution(power of 10)


B
2
1
32
1024
16384
Log.
Reciprocal virus dilution

buffer. A ten-fold and a two-fold dilution series were examined and the leaf sample used for each of these was different. The reaction time with the virus was overnight at 5°C. The number of trapped particles was calculated by counting 10 random fields per grid and using the mean of the 10 counts for comparative purposes.

b) The second part of this experiment involved a comparison of particle numbers of the two viruses U2-TMV and ToMV trapped on grids treated with each of the antisera to these two viruses. The grids were treated as in a) and the virus dilutions were from 10^{-3} to 10^{-7}. Comparative counts were obtained in the same way as in a).

Results

The effect of varying the virus concentration on the number of particles trapped is illustrated in Figs. 5/11, 5/12. The distance between, and the relative positions of, the lines at each dilution can, of course, be used as an indication of the changes in the affinity ratios shown in Table 5/7.

Table 5/7. The effect of virus concentration on the affinity ratio.

VIRUS	VIRUS DILUTIONS						
	10^{-3}	10^{-4}	10^{-5}*	10^{-6}*	10^{-7}*		
U2-TMV	0.22	1.36	0.91	0.77	1.43		
ToMV	1.63	-	0.85	1.76	2.8		
	2^{-2}	2^{-4}	2^{-6}	2^{-8}	2^{-10}	2^{-12}	2^{-14}
ToMV	1.47	1.68	1.57	1.26	1.45	2.42	1.15
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
ToMV	1.74	1.84	2.21	2.41	1.99		

* Both counts in background region.

5.5.3. *Discussion.*

In all cases except one the number of trapped virus particles decreased with increasing dilution of the virus preparation. This was

Fig. 5/11. The effect of virus dilution on the number of particles trapped on grids coated with homologous and heterologous antisera.

Grids were coated with antisera to U2-TMV (dotted line) and ToMV (solid line) and used to trap particles from a crude sap preparation of ToMV. The sap was diluted in a 2-fold (A) and a 10-fold (B) series. The number of particles trapped at each dilution was determined.

Fig. 5/11.

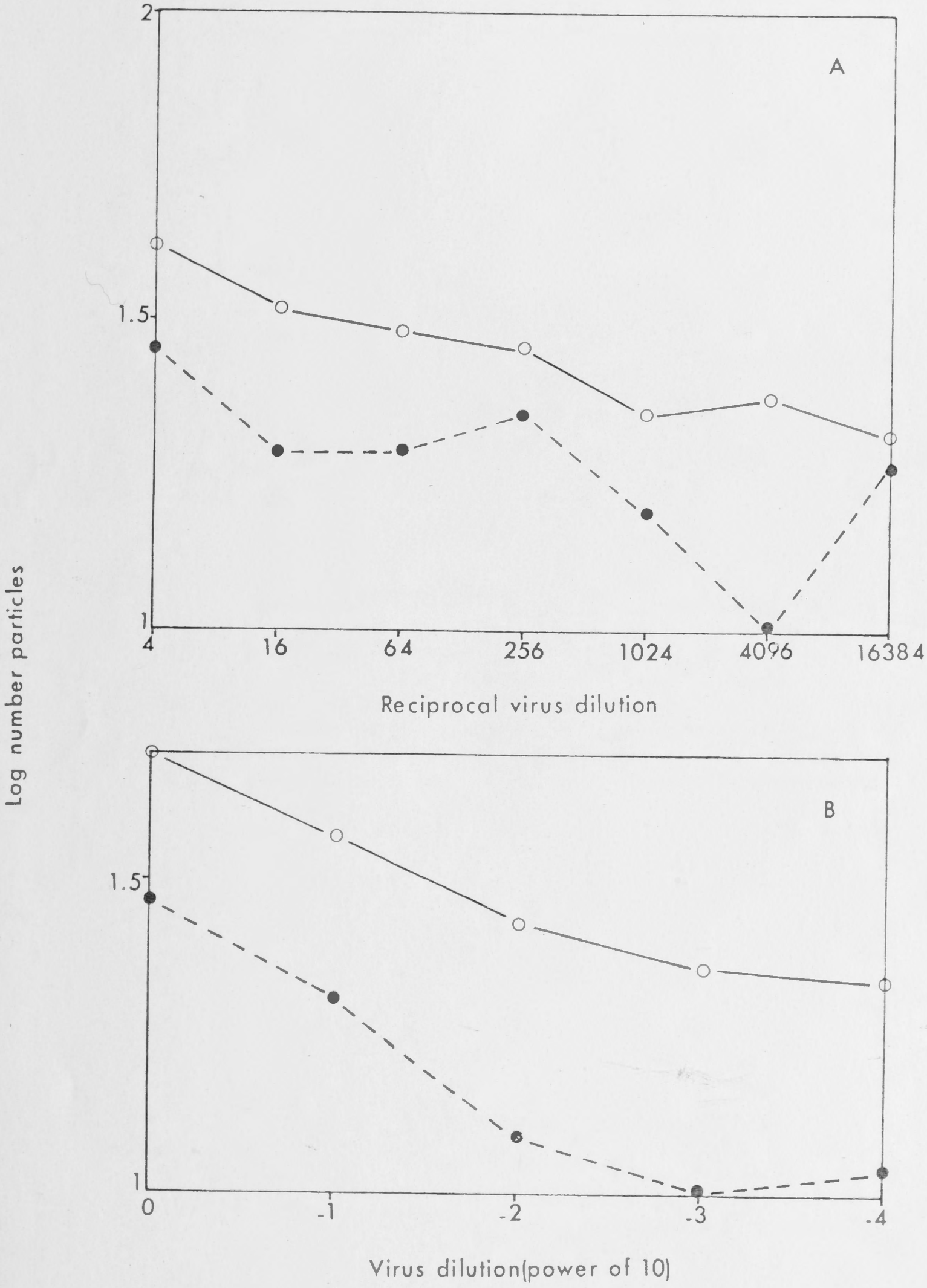
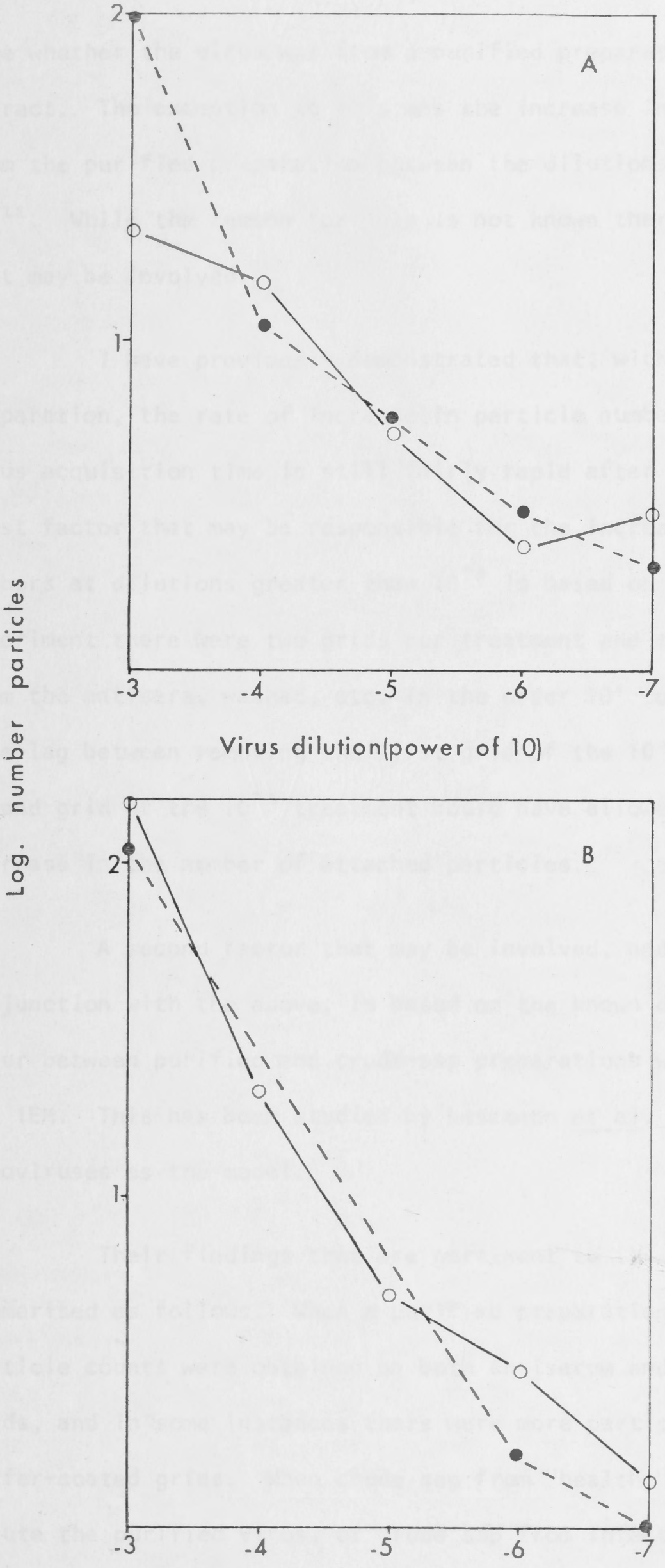

A
2
1.5
1
4
16
64
256
1024
4096
16384
Log number particles
Reciprocal virus dilution
B
1.5
1
0
-1
-2
-3
-4
Virus dilution(power of 10)

Fig. 5/12. A comparison of the particle numbers of two viruses trapped by homologous and heterologous antisera.

Grids were coated with either a.U2-TMV or a.ToMV and then used to trap virus particles from a 10-fold dilution series of a crude sap preparation of either U2-TMV (A) or ToMV (B). The homologous counts are represented by the solid line and the heterologous by a dotted line.

Fig. 5-12.


A
2
1
-3
-4
-5
-6
-7
Virus dilution(power of 10)
B
2
1
-3
-4
-5
-6
-7
Virus dilution(power of 10)
Log. number particles

true whether the virus was from a purified preparation or a crude sap extract. The exception to this was the increase in particle numbers from the purified preparation between the dilutions of 10^{-8} and 10^{-11}. While the reason for this is not known there are three factors that may be involved.

I have previously demonstrated that, with a dilute virus preparation, the rate of increase in particle numbers with respect to virus acquisition time is still fairly rapid after 90 minutes The first factor that may be responsible for the increase in particle numbers at dilutions greater than 10^{-8} is based on that fact. In the experiment there were two grids per treatment and they were removed from the antisera, washed, etc. in the order 10^{0} to 10^{-11}. Thus the time-lag between removing the first grid of the 10^{0} treatment and the second grid of the 10^{-8} treatment could have allowed a noticeable increase in the number of attached particles.

A second factor that may be involved, and may act in conjunction with the above, is based on the known differences that occur between purified and crude-sap preparations when they are used for IEM. This has been studied by Lesemann et al. (1980), using Tymoviruses as the model.

Their findings that are pertinent to this disucssion can be summarised as follows. When a purified preparation was used high particle counts were obtained on both antiserum and buffer coated grids, and in some instances there were more particles present on the buffer-coated grids. When crude sap from "healthy" plants was used to dilute the purified virus, or crude sap from infected plants were used there were very low counts on buffer-coated grids and high counts on antiserum treated grids.

The explanation offered by them was that on buffer-treated grids there are large numbers of non-specific adsorption sites that will accept virus particles. When crude-sap is present in the virus preparation the constituents of the sap compete directly for the sites and block most of them from the virus. When a grid is coated with anti-serum a large number of the non-specific sites would be blocked by the serum constituents and so only virus particles would be attached to the grid. In this case the two types of virus preparation would behave the same way.

In my experimental work with the purified virus I used anti-serum diluted 1:100. This gives fewer specific sites than would 1:1,000 and therefore more non-specific sites. It would seem from the results of Lesemann et al. that the non-specific adsorption occurs at a faster rate than does specific adsorption. This then could act synergistically with the above proposed longer exposure times of the last grids to be removed.

The third factor that may be implicated is that of the purification procedure. It is well known that high speed centrifugation can induce large aggregates of particles and some of these can be diluted as a single unit and then gradually dissociate after the dilution series has been done. There is thus the possibility that this happened during the dilutions for this experimental work.

In the experiments using crude sap extract as the source of virus there was a considerable variation in the relation between the homologous and heterologous counts. In the sets of counts from the tests where only one virus was used, the virus was identified correctly but there was a considerable variation in the value from one dilution to another and the ratios were not as high as in previous experiments comparing a.U1-TMV and a.ToMV.

The figures from the other experiment, as shown in Fig. 5/12, are very different, show little differentiation between the antisera and in many instances give a wrong identification. This was most likely due to the fact that much greater dilutions were used, and many of the counts were very low, close to or in expected background levels.

The trends shown in Fig. 5/10 are probably the most reliable as the particle numbers were much higher and none were near the expected background counts.

The variations in the ratios and the generally lower values for them are almost certainly a reflection of the fact that the viruses U2-TMV and ToMV are much more closely related than are U1-TMV and ToMV.

The results indicate that particle counts are probably best suited to more distant relationships and that, if they are used in closer relationships, care should be taken in the interpretation, and the factors that can influence numbers of particles and affinity ratios kept in mind.

5.6. Identification of viruses by trapping with different antisera

Having examined many of the factors that may affect comparative particle counts it is necessary to have a look at counts made using a range of antisera and viruses in the Tobamovirus group. As the viruses vary in relationship to each other this should give some idea of the efficacy of the method in virus identification.

One part of the experiment was designed to examine the two closely related viruses U2-TMV and ToMV using antisera diluted in CB alone and in CB plus SSC. The other part was designed to look at (a) the numbers of particles of U1-TMV trapped by 5 antisera and normal

serum and (b) the numbers of particles of each of 4 viruses trapped by each of the 4 antisera.

Materials and Methods

In the first part of the experiment the two antisera a.U2-TMV and a.ToMV were diluted to 1:1,000 in CB and a further 1:16 in SSC and used to coat grids for 90 minutes. These treatments were then used to trap viruses for 120 minutes from a 10° preparation. Trapped particles were counted on 10 fields per grid.

In the second part of the experiment the 4 antisera a.U1-TMV, a.U2-TMV, a.ToMV and a.ORSV were used at a dilution of 1:1,000 in the experiment using a single virus and 1:8,000 in the experiment with the 4 viruses. The coating time was 90 minutes. In the experiment to trap only U1-TMV, grids coated with a.CV4 and normal serum at a dilution of 1:1,000 were also used.

The viruses were all used at a concentration of 10° and the acquisition time was 4 hours for the single virus and overnight at 5°C for the range of 4 viruses.

Results and Discussion

a) Comparison of U2-TMV and ToMV

The affinity ratios were as follows:

VIRUS	ANTISERUM DILUTION	
	1:1,000 (in CB)	1:16,000 (in CB + SSC)
U2-TMV	94.5	10.5
ToMV	0.7	0.5

There is considerable variation in the figures obtained, and in the case of ToMV the identification was wrong. This is a reflection

of the closeness of the serological relationship being tested. It seems as if U2-TMV differs from ToMV in its affinity to the two antisera, and at both dilutions the identification was correct and the ratio very large. The ratio for U2-TMV at the 1:1,000 dilution is not correct as the heterologous counts were extremely low due to poor wetting of the grid, resulting in a poor preparation and very few particles.

b) Trapping U1-TMV with a range of antisera

There was a clear differentiation in the antisera, once the distance of the relationship became large enough. There was no differentiation between the more closely related ones. The figures for each of the antisera are:

a.U1-TMV	a.U2-TMV	a.ToMV	a.ORSV	a.CV4	NS
density too high to count		97.2	8.7	3.4	1.7

These results indicate that, of the heterologous antisera, U1-TMV is most closely related to a.U2-TMV, then a.ToMV, and only distantly related to the other two.

c) Comparison of 4 viruses and their antisera

The results have been calculated as affinity ratios for each of the heterologous antisera for each of the viruses and were as follows:

VIRUS	ANTISERUM To			
	U1-TMV	U2-TMV	ToMV	ORSV
U1-TMV	1.0	2.0	2.7	28
U2-TMV	0.7	1.0	2.0	25.5
ToMV	5.1	1.1	1.0	33.4
ORSV	2.7	0.04	3.0	1.0

The orchid virus (ORSV) is the most distant, serologically, of the 4 viruses. The reason for the very low value when ORSV was

tested against a.U2-TMV is not known but is most probably due to the occurrence of an aberrant trapping like that occasionally reported in the literature, and recorded during my work, due to some of the "unknown factors" that seem to be part of the problem associated with virus particle counts. I have not recorded this type of ratio with two viruses as serologically distantly related as ORSV and U2-TMV.

The results confirm those obtained in b), and, because of the lower numbers of particles being present show the relationship between the antisera to U1-TMV and U2-TMV and the virus U1-TMV.

It is evident that the apparent relationships shown in reciprocal tests varies. This can happen to a minor extent due to real variations that exist when a serological test is conducted between 2 viruses and their antisera. There are also many other factors causing variations of differing magnitudes, including variations between individual rabbits. Some of these factors are discussed by van Regenmortel (1975).

5.7. General Discussion

The experimentation reported in 5.2 - 5.6 can be considered, in broad terms, to cover 3 main areas: (1) the coating antiserum (2) the virus preparation (3) the use of the method for diagnostic purposes. This discussion will therefore be sectionalised under those headings.

5.7.1. *The coating antiserum.*

There are 3 buffers that seem to be favoured as an antiserum diluent, Tris (Derrick, 1973b; Derrick and Brlansky, 1976; Paliwal, 1977); phosphate (Milne and Lesemann, 1978; Lesemann, et al., 1980; Roberts et al., 1980); carbonate (Thomas, 1980; Plumb, pers. comm.).

In all instances the particular buffer is used without comment as to the reason for its selection and it seems as though each laboratory uses what it "feels" to be the best. In general my preliminary experimentation led me to agree with Plumb that carbonate buffer seems to be better than others and was always used in my experiments.

While there has been no published report on coating antiserum, there has been some work done on antiserum used to "decorate" particles that are present on a grid (Milne and Luisoni, 1975, 1977). They found that saline in the buffer resulted in a poor final image and that other buffers offerred no advantage over phosphate. Because of this they used phosphate buffer for diluting the antiserum whether it was to be used for coating grids or decorating virus particles.

In my tests with antiserum concentration, the lower dilutions (less than 1:1,000) were not considered because it has been found that particle numbers are lower when more concentrated antisera are used.

In the early work on IEM, high concentrations of antisera were used (Derrick, 1973b; Milne and Luisoni, 1977). Soon after the initial work by Derrick, he and Brlansky (1976), when working with a Potyvirus, found that greater dilutions were better. Subsequent experimentation has shown this to be true for the Reoviruses (Milne and Lesemann, 1978), Luteoviruses (Paliwal, 1977), Tymoviruses (Lesemann, Bozarth and Koenig, 1980), and Tobamoviruses in this research project.

In general it was found that particle numbers were better at a dilution of about 1:1,000 but that high numbers were retained with much greater dilutions (up to 10^{-7} in the case of the Tymoviruses). It was because of this that most antisera are used initially at a

dilution of about 1:1,000. The fact that these antisera all worked best at this dilution, but had titres that varied greatly, shows that the best dilution is not related to the titre.

It is most likely that at dilutions less than ca. 1:1,000 the decreased trapping ability is due to the presence of competitive inhibitors that occur in serum. Milne and Lesemann(1978) consider that the inhibitors are due to other serum proteins competing for sites on the grid with the specific IgG. The competitor is most probably not non-specific IgG as Milne and Lesemann (1978) added various concentrations of both normal serum and bovine serum albumin to the antiserum and both had very similar effects on the particle numbers. They considered that the albumin in serum could account for the effect.

It is unlikely that non-specific IgG would be inhibitors as the use of protein-A by Shukla and Gough (1979) overcomes the effect of any inhibitor. This is also shown in section 3.

It is because the inhibitors are not IgG molecules that NS can be diluted and used as a diluent for specific antiserum to cause a decrease in particle numbers. The non-specific IgG is acting as a competitor but, by the effects noticed, the amount of IgG that does act as an inhibitor must be low and so the decreases in particle numbers are small and not readily reproducible.

My findings that the use of antiserum dilution series are not suitable for diagnostic use is a result of the fact that, once the inhibitor is diluted out of an antiserum, the grid coating ability is not really a concentration dependent phenomenon over quite a substantial range of dilutions. Derrick and Brlansky (1976) demonstrated that they could still trap substantial numbers of particles of potato virus Y with antiserum diluted 1:320,000 and 1:3,200,000.

The reason for this would appear to be that even in very dilute antisera there are sufficient IgG molecules to occupy the available sites in the 10 minute coating time. It would therefore seem that it is only when the dilution is too large to have sufficient IgG molecules to fill the sites in 10 minutes (e.g. very low titre sera at 1:1,000) that there may be an advantage in increasing the coating times.

The adsorption time that is allowed in various methods varies from 5 minutes to 90 minutes. In no instance has any reason been given for the choice of time. My results demonstrate that for a given antiserum dilution the coating time plays little part and it seems as though the coating is completed within the first 10 minutes. It may well be that in the published methods the shorter coating times may have been based on some experimental knowledge but the longer times appear to have no basis other than using a longer time to "play safe".

It may well be thought that, under some exceptional circumstances, such as when there was a very low concentration of specific IgG in the original antiserum, that there may be some advantage in increasing the coating time. Under almost all circumstances there seems to be sufficient IgG present at a dilution of 1:1,000 to coat the grid within 10 minutes.

As shown in section 3 the inhibitors in serum are overcome by precoating the grids with protein-A and under these conditions the antiserum has then to be used at a higher concentration (ca. 1:20) and the efficiency falls off at greater dilutions. In this section the use of protein-A caused a steady decline in trapped particle numbers with dilution and offers a better method of being able to determine a serum end-point for diagnostic purposes. This affects the numbers

trapped on heterologous antiserum more than those on homologous antiserum and so the differentiation between two antisera in picking up a virus becomes more pronounced and would aid diagnosis.

5.7.2. *The virus preparation.*

The virus concentration and virus acquisition time are closely inter-dependent and a decrease in one or the other leads to a decrease in virus availability. This is very important from the point of view of virus detection in plants in which the virus concentration cannot be controlled and varies from reasonably high to very low. The big advantage that IEM offers is the ability to work with crude sap extracts and so this must be considered.

As the grid-coating step offers virtually no possibilities for manipulation to control the sensitivity of detection, control of the virus acquisition step is the only way in which this can be done. Because the concentration of the virus in sap is low for many virus/host combinations the only alternative left for the lower concentration viruses is to alter the reaction time of the coated grid with the virus preparation. With very-low-concentration-viruses this frequently necessitates an overnight reaction, then low temperatures or a high humidity are also essential to avoid evaporation of the drops of the virus preparation. The effect of virus acquisition times on particle trapping in this and other studies is discussed in greater detail in 5.3.3.

5.7.3. *The diagnostic use of particle trapping.*

The method can be used diagnostically provided that attempts are not made to separate viruses, or antisera to viruses, that are too closely related. Certain of the factors that contribute to the result must also be kept in mind when analysing results from comparative counts.

A distinction should be made between the true relationship that exists between two viruses and the ability to maximise any difference between them to make diagnosis easy, straightforward and not relying on a detailed statistical analysis of the results to determine differences. When using IEM diagnostically it is best to maximise any differences.

One way of maximising the differences is to reduce the chance of contact of the virus particles and specific antibodies so that not all available sites are occupied. There are fewer cross-reacting than homologous antibodies in an antiserum, and a reduction in chance contact affects the heterologous more than the homologous counts, thus creating a larger difference between the two than would occur if all sites were occupied. Lesemann et al. (1980) noticed that counts of particles tended to plateau when they examined the effect of virus concentration on particle trapping. They referred to the occupation of all available sites as "saturation". Using this terminology it is preferable, therefore, to operate at conditions of under-saturation for diagnostic purposes.

Roberts et al. (1980) attempted to establish relationships in the Luteoviruses by using 11 antisera, obtained from a number of countries, to trap particles of a local virus isolate. They used acquisition times of 60, 120, and 240 minutes and found the best differentiation was by using a 60 minute time. Although they do not comment on this, it is an illustration of operating at under-saturation, but above background, levels.

An important principle demonstrated by my experimental work is that when comparisons are made it is more reliable to compare one virus with a range of antisera than to compare a number of viruses

using one antiserum. I have shown that the number of particles trapped on a grid is directly dependent on the concentration of particles in the virus preparation. Thus when a number of virus isolates is compared using one antiserum a lot of the differences in particle counts could be directly attributed to the virus concentration in the starting preparation. There are reports in the literature that would also indicate this.

Various isolates of barley yellow dwarf virus have been compared using a single antiserum to one known strain (Paliwal, 1977). Doing this it was possible to obtain a detection and partial identification but the author did note that the mean counts may not have really been comparable as the amount of virus could vary significantly between different isolates even when propagated in the same host cultivar under the same environmental conditions.

Another phenomenon that can affect the diagnostic use of virus particle counts was recorded for Belladonna mottle virus, a member of the Tymoviruses (Lesemann et al., 1980). They used purified preparations of a range of viruses from the group, all at the same concentration of 5 µl/ml, and a range of antisera to trap particles. All antisera seemed to trap more particles of Belladonna mottle virus than would be expected, and in many instances more than the number of homologous particles. They concluded it was due to greater avidity of this virus for antisera. It could be seen from their results that this factor militated against comparing a number of viruses using an antiserum to one of them. I suspect this happened in some of my work and resulted in some of the apparently wrong diagnoses.

In the same paper on Tymoviruses a comparison was made, using particle counts, of 4 different Tymoviruses and 7 antisera to different

viruses in the same group. If the results in their Table 2 are studied it can be seen that comparing 1 antiserum with a number of viruses frequently gives the wrong result, due mainly to the fact that one virus has a greater tendency to be trapped than the others. When the same results are examined by comparing one virus with a range of antisera then the results are much more meaningful and agree with previous detailed studies made on the relationships of this group of viruses (Koenig and Lesemann, 1979).

This type of result confirms my findings that the comparisons should really only be made on a range of antisera, even with purified preparations with the same starting concentrations. Thus there are factors other than virus concentration involved and avidity of a virus for antisera is certainly one of these.

The involvement of other factors is also indicated in a study of oat sterile dwarf virus (Milne and Lesemann, 1978) in which they found some differences in particle counts that they thought did not reflect serological differences but rather "various factors not well controlled or understood in applying the method", something that probably occurs with <u>all</u> methods used in biology.

6. GOLD-LABELLED ANTIBODY DECORATION

"Read not to contradict and confute, nor to believe and take for granted ... but to weigh and consider."

Bacon.

6.1. Introduction

The field of immuno-electron microscopy combines some of the advantages of two major methods of virus detection and identification: electron microscopy and serology. While greatly increasing the ability to detect viruses in infected plant tissues it suffers from the disadvantage of not satisfactorily differentiating between strains of a virus.

Milne and Luisoni (1975) found that, once virus particles were on a grid, antibodies could be added and they formed a "halo" around the virus particles with which they reacted. They termed this process "decoration". The disadvantages of this method seem to me to be (1) a dilution series of antisera has to be made for end-point determination and then, for a particular set of viruses, a suitable dilution can be determined that will give a diagnostic differentiation; (2) light decoration is difficult to detect and can sometimes appear similar to poor staining or other faults in preparation; (3) the "halo" cannot be readily identified as being due to IgG molecules. *

The last of these points has also been a problem in immunocytochemical studies of sectioned material and antibodies have had to be labelled to avoid any ambiguity and to enable better visualisation of any antigen - antibody reaction that may take place. This was especially so when any antibody labelling was too light to show up as a darker staining region around the antigenic material.

To overcome these problems in sectioned material, electron-opaque markers were attached to the IgG molecules. Two types of markers have mainly been used, ferritin and heavy metal colloids (of which gold has been the most successful).

* see Addendum, Plate 2.

The development and use of ferritin has been reviewed by Morgan (1972) who considered that the method had a number of drawbacks, the principal ones being (1) the overall size was not suitable for some sectioning work; (2) after conjugation little more than 10-20% of the antibody is labelled; (3) there is always a large excess of non-reactive ferritin conjugates that lead to a high background level and make specific labelling hard to identify; (4) conjugation results in a considerable reduction in titre; (5) the method does not allow the sections to be labelled satisfactorily, the labelling being done on the tissue prior to sectioning.

The field of immunocytochemistry has been reviewed and discussed in detail by Sternberger (1979). In this book he discusses other antibody markers that could be used to give enough contrast in the electron microscope provided a large number of heavy atoms can be concentrated into a restricted domain and the resulting particle conjugated to an immuno-globulin. These criteria can be met by colloidal gold.

Colloidal gold is a negatively charged hydrophobic sol, the stability of which is maintained by electrostatic repulsion. The colloid is sensitive to electrolytes and their addition alters the electrochemical environment resulting in immediate precipitation of the particles. The particles can however be stabilised against this by some macromolecules including proteins and carbohydrates. One species of protein that binds firmly to colloidal gold is that known as immuno-globulins and this fact can be exploited to use gold to label tissue that has to be treated with specific antibodies.

The first use of such a technique was by Faulk and Taylor (1971) and then later with some modifications, by Romano et al., (1974).

In these and other studies the colloidal gold was used as a direct label onto antibody IgG that was adhering to sites of the specific antigen in the tissues being studied.

The method was simple to use and had a number of advantages over the older method of ferritin labelling: (1) preparation was simpler; (2) the marker did not have to be conjugated with specific antibody prior to use; (3) the diameter of the colloidal gold particles can be controlled during preparation and when used at about 50 nm was about twice that of ferritin; (4) the electron opacity is much higher than that of ferritin because of the size, because Au is heavier than Fe and because the whole diameter of the particle is filled with Au, but only the inner mycel of ferritin contains Fe.

Even though the method was simple and seemed to work well it had a serious limitation in that not all species of immuno-globulin would bind firmly to colloidal gold and, unfortunately, among those that would not bind firmly were human and rabbit IgG, two species that were used quite frequently for serological work.

There was another protein that had been used in serological work, mainly because of its inter-action with immuno-globulins, an aspect that was investigated by Forsgren and Sjöquist (1966, 1967). This protein was protein-A (pA) and was isolated from the cell walls of Staphylococcus aureus. Forsgren and Sjöquist (1966) noted that the interaction of pA was principally with the Fc (non antigen-reacting) end of the human IgG molecule. They then examined (1967) the reaction of protein-A with rabbit IgG and found that this reaction also seemed to involve the Fc end of the molecules. They did not observe any reactions between pA and the Fab fragments with normal rabbit IgG or rabbit anti-pA, but conceded that some soluble complexes may have been

formed but were not detectable by the method used.

This work involved the two antibody types that colloidal gold would not react with. It seemed, therefore, that use could possibly be made of pA to form a link between specific antibodies conjugated to antigens in tissue and the colloidal gold markers, providing protein-A would react with colloidal gold.

To overcome problems of labelling human and rabbig IgG with colloidal gold Romano and Romano (1977) investigated two possibilities: (a) the chemical modification of IgG to render them suitable for labelling with gold and (b) the reaction of gold particles with pA. The results of the first possibility were not satisfactory. In regard to the second alternative pA was found to form a stable complex with gold particles and this complex was successfully used to label surface antigens in a range of situations. The method had a number of advantages over the use of ferritin (1) there was no covalent bond between the gold and the protein-A; (2) preparation of the reagent is very simple; (3) there is no need to purify the antibody to the specific antigen (i.e. cells could be incubated with whole antisera).

There is at present little information on the combining ratios of the 3 principal reactants. There does not appear to be any figures available on the number of pA molecules per gold particle. Reports of the combination of IgG:pA vary. In theory one molecule of pA is able to react with a maximum of 4 Fc portions (Roth et al., 1978), but in practice it seems as if it only reacts with 2 Fc portions (Sjöquist et al., 1972). As a result the method cannot yet be used for accurate quantitative work.

Since that time there have been 3 main developments in the use of colloidal gold to label antibodies and these are (1) the

detection of intracellular antigens in animal tissue (Roth et al., 1978) previously only surface antigens were detectable (2) Craig and Millerd (1981) used the method on plant tissue to detect storage proteins (3) the method was modified for use in detecting and identifying plant viruses in liquid preparations (Pares and Whitecross, in press).

One of the major uses of specific antibodies has been in the serological detection and identification of viruses. An attempt had been made to utilise ferritin - conjugated antibodies on purified preparations of a virus but was unsuccessful because of the large excess of non-specific conjugated globulin. This problem was partly overcome by Singer and Schick (1961) who precipitated tobacco mosaic virus (TMV) with unlabelled antibody, dissociated the complex, conjugated the resulting antibodies and then used this for labelling suspensions of TMV. This method had obvious disadvantages such as requiring large amounts of purified virus to obtain sufficient antibody and can necessitate too much work to make it a practical method for virus diagnosis. Since then there does not appear to have been any more attempts to use labelled antibody for diagnosis of viruses in suspension.

Plant viruses can also be examined in the electron microscope by crushing a small piece of infected tissue in negative stain and drying the preparation on a grid. Providing the virus is present in a reasonably high concentration in the host tissue then virus particles can be readily seen in the electron microscope. The development of IEM greatly improved the method and has resulted in the detection of viruses that occurred in too low a concentration to be detected by ordinary negative staining.

One of the significant recent developments was the technique of decoration (Milne and Luisoni, 1975) as mentioned earlier and has

become a very useful diagnostic tool. A comparison of the technique used by Roth et al. (1968) and the technique of Milne and Luisoni (1975) showed some similarity between the application of specific antibodies to sections and the application of antiserum to virus particles on a grid. It therefore seemed feasible that the gold-labelling technique could also be used, or a suitable technique could be developed, on decorated virus particles.

The following experiments were undertaken to examine such possibilities, along two lines (1) to examine the possibility of adding a gold label to decorated particles and (2) to examine the suitability of combining the gold label and the antibody before application to the virus particles. The first of these two will be referred to as "decoration and label" and the second has been developed during my research program and has been termed "gold labelled antibody decoration" (GLAD). The technique has been described in a short preliminary paper that incorporates part of the experimental work described in section 6.3.2. Fig. 1 of the paper is the same as Plate 6/1 in this thesis. The paper has been accepted for publication by the Journal of Immunological Methods and a copy of the manuscript is enclosed at the back of this thesis.

6.2. Decoration and Label

The preliminary experiment was done by trapping U1-TMV on a grid, decorating the particles with homologous antiserum and then applying a preparation of gold/protein-A (G/pA) for 45 minutes, washing the grids in 0.03 M phosphate buffer and negatively staining in ammonium molybdate. There were some grids untrapped and some undecorated. All combinations of trapping and decoration had large numbers of G/pA particles present* which indicated that the method may

* see Addendum, Plate 3.

not be too successful. Counts were also made of the number of G/pA particles attached to each virus particle and there was a mean of 2.6 per decorated particle and 0.35 per undecorated particle.

Although there was this difference, it could not be detected until detailed counts were made and the very high background levels made this job difficult, and not what was required for a useful diagnostic method.

Because of this a series of detailed experiments were made to see whether the method could be improved, or the background reduced. The aspects examined were (1) precoating grids with pA (2) decreasing the virus density (3) using untrapped preparations of virus (4) varying the label application times from 5-120 minutes (5) decorating with a range of different antisera including normal serum (6) comparing 2 viruses and their antisera and (7) varying the dilutions of the G/pA from 1:2 - 1:200.

In general these experiments yielded little information that would enable any improvement in the technique for diagnostic use. One point that did come out of all of these experiments was that G/pA at dilutions of 1:2 and 1:4 gave a coverage of gold particles but at greater dilutions there was very little gold present.

The main problem with labelling the antibodies in this series of experiments centres around the non-specific labelling over the whole area of the grid and it is not until detailed counting of particles attached to viruses that any differences show up. There is obviously a need to develop a method to increase the association with the virus particles and/or decrease the non-specific attachment to the grid surface. One possibility of doing this was to conjugate the antibodies

with the G/pA before it is applied in the decoration step, and this method is examined in the following series of experiments.

6.3. Gold Labelled Antibody Decoration (GLAD)

In the experiments described in this section, the starting point will be a grid with a formvar/carbon film and virus particles trapped by homologous antiserum. In some work with two viruses the trapping was by a mixed antiserum and it will be stated when this is applicable.

Gold-protein-A was mixed with the antisera in the ratio of 1:2 (vol:vol) unless specified otherwise. For the mixed preparation the G/pA was used at a dilution of 1:2 and the antiserum at 1:1,000. The gold particle was approximately 18-20 nm diameter. The G/pA and antiserum were mixed thoroughly and left standing ca. 1 hour before use.

To avoid long terminology the conjugate of G/pA and an antiserum will be referred to by the notation G/antiserum. As an example G/pA conjugated to the antiserum against U1-TMV will be referred to as G/a.U1-TMV.

The conjugate was applied by floating a grid on a 5-10 µl drop for a specified period of time (usually in the region of 45-90 minutes). The grid was then removed, washed by 20 drops 0.03 M PB and 6 drops of 2% ammonium molybdate, dried, and examined in the electron microscope.

6.3.1. *Preliminary experimental work.*

Experimental

As a first test of this variation in labelling, U1-TMV was decorated with G/a.U1-TMV, G/a.U2-TMV, G/a.CV4-TMV and G/normal serum (NS).

On examination the two grids with the closest related antisera (a.U1-TMV and a.U2-TMV) had gold particles apparently evenly spread over the surface and there appeared to be some association with the virus particles. The other two treatments had almost no gold particles on them.* This is in direct contrast to the results from decoration followed by labelling in which there was virtually no difference between related and unrelated antiserum treatments, even NS decoration having a high number of gold particles present. The appearance of the grids can be seen in Plate 6/1.

Because of the apparent success in using labelled antiserum as opposed to decoration and labelling another experiment was set up to compare GLAD and decoration and labelling using U1-TMV and the 2 antisera a.U1-TMV and a.CV4-TMV. Again both of the decoration and labelling treatments had an even distribution of gold over the surface but in the GLAD treatment only the homologous antiserum treatment had a good distribution of gold over the surface, there being virtually no gold present in the G/a.CV4-TMV treatment.

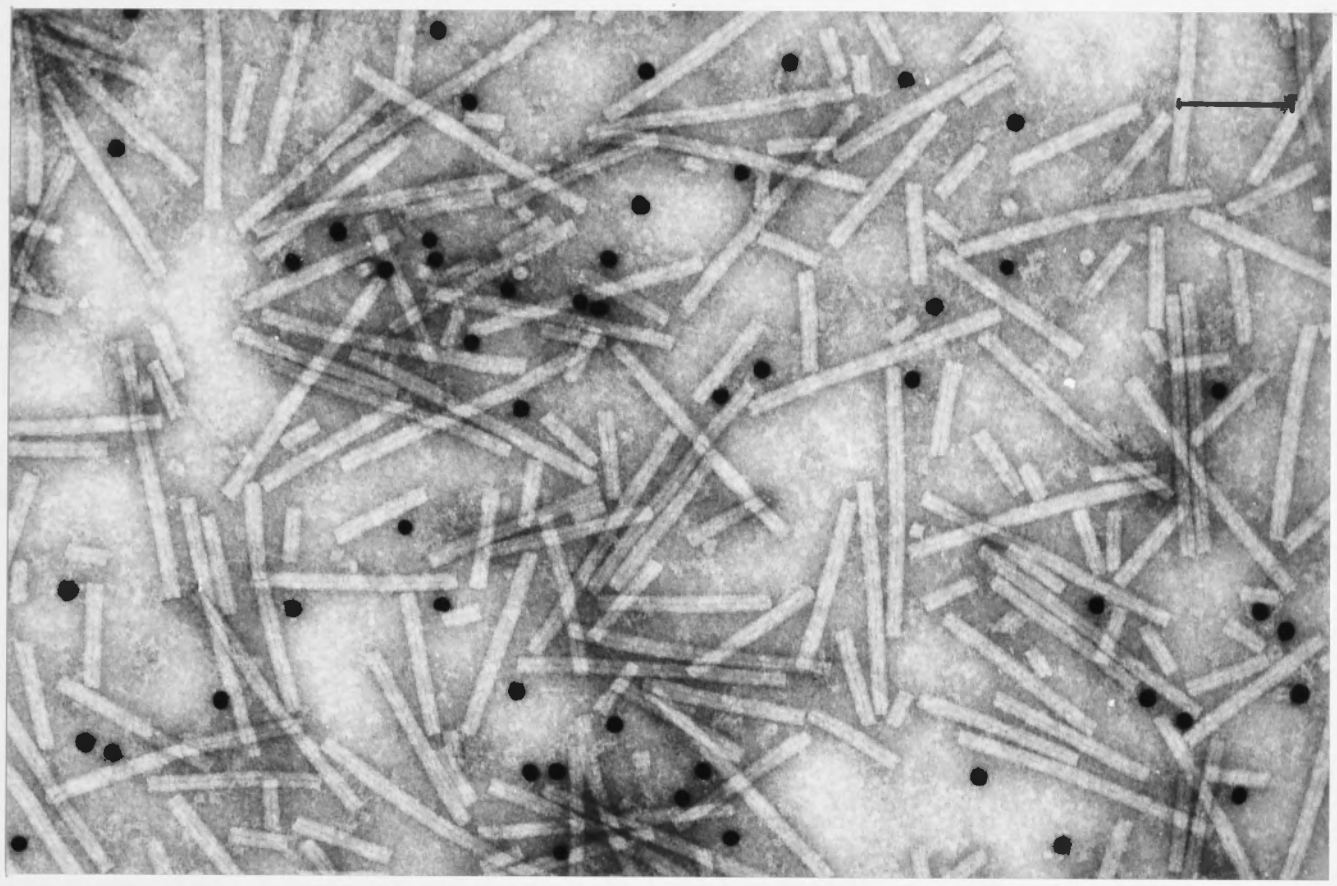
The ratio of mixing G/pA and antiserum (1:2) was arbitrarily chosen but nevertheless gave a good result. A test was then made to see what variation in counts would be given if a range of conjugating ratios was used and the following were tested 2:5, 1:2, 2:3, 1:1, 2:1, and counts were made on 10 random binocular fields on each, the means of the 10 counts being 7.1, 6.4, 5.9, 8.6 and 13.1 respectively.

All viruses can vary in concentration due to the stage of infection at which the sample is taken. At some stages the concentration is very low and some viruses are only present at these low concentrations. This variation in concentration may affect the homologous and heterologous counts and comparisons. Additionally G/pA may be useful in detecting the presence of a virus more readily than by searching for particles.

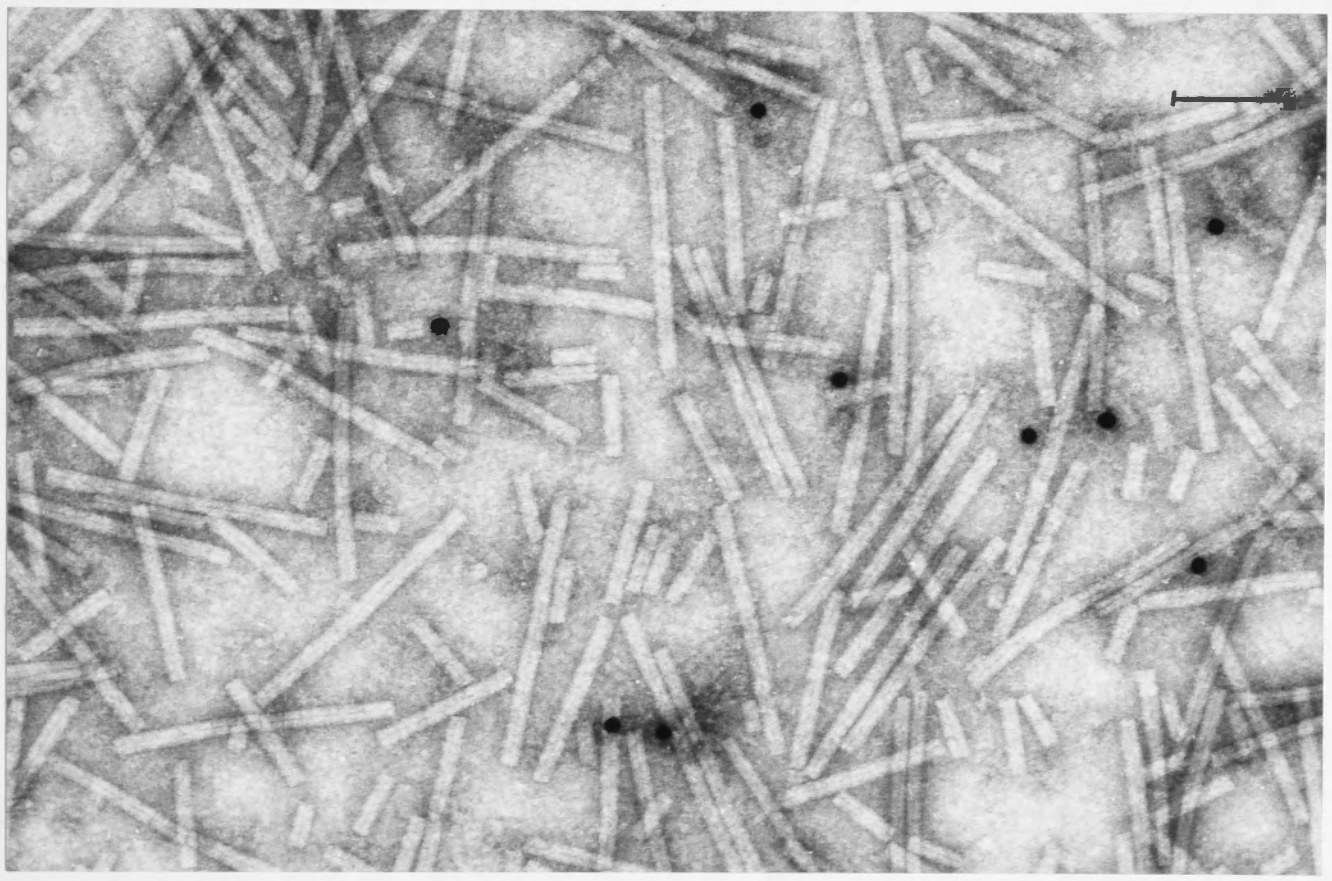
* see Addendum, Plate 3.

Plate 6/1. The deposition of gold particles on a preparation of U1-TMV treated with gold conjugated to the homologous antiserum (A) and conjugated to normal serum (B). The bar on each micrograph represents 100 nm.

A



B



A brief examination was made of these two points.

The first test was done using an isolate of ToMV that was in an early stage of infection and the sap extract was diluted in a 4-fold dilution series from 1:64 - 1:4,096. Virus particles were quite rare at all dilutions except 1:64, but when tested with G/a.ToMV the counts of G/pA were very low but did not vary much, the counts being 3.7, 3.5, 3.0 and 2.8 respectively.

The second trial used a preparation of ToMV diluted to 10^{-1} and 10^{-3} and then treated with G/a.U1-TMV and G/a.ToMV. Comparison of homologous and heterologous labelling is shown by the affinity ratios of 4.7 (mean of 4) for 10^{-1} and 3.6 (mean of 36 ratios) for 10^{-3}. In both instances the standard error of the mean was ca. 0.2.

A more comprehensive test was then made of the latter point using ToMV diluted to 10^{0} and 10^{-5} and labelling with G/a.U1-TMV and G/a.ToMV, 3 grids for each treatment. For each grid 2 sets of counts, each on a different 10 random binocular fields were made and the mean of each 10 calculated. This gives 2 counts per grid and 6 per treatment and each of the 6 was used to calculate the affinity ratios with each of the other 6 counts, giving 36 ratios per virus dilution. The mean of the ratios was 8.3 (±0.7) for 10^{0} and 2.0 (±0.04) for 10^{-5}.

Discussion

Despite the discouraging results from the use of decoration followed by labelling, the first two of these preliminary tests of the GLAD method shows that there is some promise in the use of labelling the antibodies prior to use for decoration and that with distantly related antisera or viruses the result can be assessed visually without any need to use detailed counts or statistical analyses.

There is some indication that it may be possible to use the method to aid detection of viruses that are present at such low concentrations that very few particles can be seen.

Although the two experiments examining the gold particles present using homologous and heterologous antisera cannot strictly be compared, in the sense that the virus preparations in both were not from the same dilution series, the starting point for both was infected leaves from the same plant. There is, therefore, probably some valid reason for being able to compare them, especially since the results from the two different experiments follow a pattern. This is seen in the following summary of the ratios for the 4 dilutions of ToMV. The figures are the means of all the ratios for each treatment (standard error of the mean): 10° - 8.3 (0.7); 10^{-1} - 4.7 (0.2); 10^{-3} - 3.6 (0.2); 10^{-5} - 2.0 (0.04).

These figures show a decrease in the affinity ratio as the density of virus particles on the grid decreases. Heterologous counts are always closer to background levels than homologous counts and therefore the rate of decrease in heterologous counts is also less until each reaches the background level and finally the affinity ratio should approach unity. Because of the very low numbers of gold particles in background counts, the ratio at this level would fluctuate greatly, probably hardly ever be 1.0, and would be meaningless. This is because the counts for homologous and heterologous decoration would rely entirely on the chance of seeing any particles in one or other of the preparations.

Thus for differentiation between two antisera to be maximised higher virus densities on the grid appear to be necessary, a factor that would be important where two closely related viruses are being studied.

When the gold:antiserum combining ratios were examined there was very little difference in counts when the ratio was less than 1:1, but when there was a 2:1 combining ratio there was an increase of ca. 1.9x in the counts. This could have been due to either a rapid increase in the conjugation of the two components or an excess of unconjugated G/pA that would attach non-specifically to the preparation as it does when applied after decoration. Because there was no evidence of the counts increasing at the other dilutions it seems more likely that the second explanation is the more applicable in this instance. Because the method seemed to work adequately in these experiments at a combining ratio of 1:2 it was decided to use this in subsequent experiments.

6.3.2. *Utilisation of GLAD in comparisons of Tobamoviruses.*

In the light of the results from 6.3.1, it seems as if the method may be useful in diagnostic work with viruses and would offer some advantages over particle counting. One of these advantages is that shown by decoration alone and that is, in theory at least, a result can be obtained from a sample in which there is so little virus that only a single particle can be found on the grid. In practical terms it means that much smaller samples and much lower virus concentrations can be worked with.

In this section experiments are reported that examine 2 pairs of more distantly related viruses (U1-TMV and ToMV; U1-TMV and ORSV); two closer related viruses U2-TMV and ToMV; 3 Tobamoviruses and their antisera, U1-TMV, U2-TMV, ToMV.

a) <u>More distant relationships</u>. Previous preliminary tests have shown a reasonable separation of two viruses such as U1-TMV and ToMV or ORSV. In this section this is examined in more detail but the

work is also designed to examine other facets of the technique as well.

Materials and Methods

The two viruses U1-TMV and ToMV were used to test diagnostic separation firstly by using G/a.ToMV at a combining ratio of 1:3 and then by using GLAD with the two homologous antisera and some partially cross-absorbed antisera that had had some of the cross-reacting antibodies removed. In a third experiment the two viruses were trapped onto separate grids by a mixed antiserum of equal parts a.U1-TMV and a.ToMV, each 1:1,000. This ensured that for comparative purposes all grids had the same antiserum coating. For each virus 5 grids were treated with G/a.U1-TMV and 5 grids with G/a.ToMV, and counts made on 20 random fields per grid, the means of the first and second group of 10 counts taken for each, giving 10 means per treatment from which 100 affinity ratios could be calculated per virus. The mean of the 100 ratios was used for comparisons.

Finally the two viruses U1-TMV and ORSV, which are more distantly related than are U1-TMV and ToMV, were trapped onto grids by a mixture of the two homologous antisera. The two viruses were mixed, prior to trapping in the various proportions of (U1:OR) - 1:10, 1:4, 1:2, 1:1, 2:1, 4:1 and 10:1. Crude sap preparations in phosphate buffer were used. Each of these grids was then treated with G/a.U1-TMV, 10 counts made per grid, the mean being used for comparative purposes.

Results and Discussion

In the preliminary trial using only one antiserum, the conjugated labelling gave a correct identification of the two viruses and counting was not necessary to determine any differences.

When the two viruses were compared using each of the homologous antisera and some partially cross-absorbed antisera the results were as shown in Table 6/1.

Table 6/1. Affinity ratios using the GLAD technique in diagnosis of U1-TMV and ToMV.

VIRUS	ANTISERA	
	Whole	Partially cross-absorbed (a)
U1-TMV	1.0	21 (6.5)
ToMV	6.6	8 (2.3)

(a) A repeat of this part of the experiment was done using a shorter virus acquisition time and the results from this are shown in brackets.

As can be seen from these results the only instance of doubtful diagnosis was in the case of U1-TMV tested with gold/whole antiserum, indicating that this virus has a greater affinity for a.ToMV than ToMV does for a.U1-TMV. In this respect U1-TMV and a.ToMV react like closely related antisera and so partial or complete cross-absorption of the antisera should have an effect on this reaction more than on the other 3 reactions. This is, in effect, what happens as can be seen in the figures for the partially cross-absorbed antisera. The ratio for U1-TMV is greatly changed but that for ToMV is changed very little.

In the third of these experiments in which 100 ratios were calculated for each of the two viruses, the ratios were 0.97 for U1-TMV with an actual range of 0.51-1.63 and the corresponding figures for ToMV are 5.56 (4.55-6.25), confirming the previous results from the whole antisera. The figures for ToMV also fit into the range given in the preliminary experimentation for virus dilutions from 10° - 10^{-5}.

When various mixtures of U1-TMV and ORSV were tested with G/a.U1-TMV, the following counts were given - the ratios on the top

line are the volumes crude sap extract U1-TMV : vols crude sap ORSV:

1:10	1:4	1:2	1:1	2:1	4:1	10:1
2.3	2.4	2.9	3.1	5.6	4.9	5.2

The mean counts seem to fall into 2 groups, those in the region of 1:10 - 1:1 and those with more U1-TMV present (2:1 - 10:1). The variation is not very great from one extreme to the other - about 2.25x. This is, however, probably all that could be expected as in the preliminary experiments a change of virus concentration from 10^{0} to 10^{-1} altered the homologous counts by a factor of 1.3x and a concentration change from 10^{-1} to 10^{-3} altered the counts by a factor of 1.8x. The individual dilution steps are probably too small to show any significant change from one to another and it is only the overall change that is noticeable.

b) <u>More closely related viruses</u>. The two viruses U2-TMV and ToMV are quite closely related serologically and have proved difficult to differentiate by particle counts from trapping onto grids coated with the two antisera. These were therefore selected to examine the use of GLAD to separate two closely related viruses and their antisera.

<u>Materials and Methods</u>

The two viruses were compared firstly using whole antisera conjugated to G/pA and then in another experiment using partially cross-absorbed antisera to compare the two viruses by particle trapping, decoration and labelling and GLAD.

In the experiment using whole antisera the gold particles were counted by taking the mean of 3 transects per micrograph from each of 3 micrographs for each of 5 grids per treatment. The counts done in the electron microscope were of 20 random fields per grid (5 grids per treatment).

The two partially cross-absorbed antisera were a.U2-TMV that had some cross-reacting antibodies removed by ToMV and a.ToMV that had been similarly treated with U2-TMV. These two antisera were used to (a) trap virus particles (b) decorate virus particles that were then treated with G/pA and (c) conjugate with G/pA and then used in the GLAD method. In all 3 comparisons were made by using the mean of 10 counts per grid.

Results and Discussion

In all experiments the affinity ratio will be used for each virus. When whole sera were used the ratios were:

U2-TMV - 2.5 from micrographs, 2.8 in microscope.
ToMV - 0.8 from micrographs, 0.9 in microscope.

This indicates a good agreement between the two methods of counting and shows that ToMV has a greater affinity for a.U2-TMV than does U2-TMV for a.ToMV. The ratios, when compared to those obtained for U1-TMV and ToMV show that U2-TMV and ToMV are more closely related.

In the previous work using U1-TMV there was a similar situation in which one virus had a greater affinity for the heterologous antiserum and when partially cross-absorbed antisera were used the differences become very apparent. If this is so then the use of partially cross-absorbed antisera should change the figures for ToMV above from ca. 0.9 to a much larger figure.

The results from cross-absorption are:

(i) Particle trapping: U2-TMV 0.39; ToMV 30.23.
(ii) Decoration + Labelling: U2-TMV 0.56; ToMV 0.47.
(iii) GLAD: U2-TMV 1.30; ToMV 16.70.

It would appear from this that GLAD seems to be a more reliable method than particle trapping for diagnostic purposes.

Decoration and labelling, because of the high rate of non-specific attachment is the least suitable method. The GLAD results show the same trend in that the virus that did not differentiate between 2 whole antisera shows a pronounced difference when partially cross-absorbed antisera are used.

c) Comparison of 3 viruses and their antisera. The three viruses U1-TMV, U2-TMV and ToMV were all treated with G/pA conjugated to each of the antisera to the viruses. Counts were made on 20 random fields in the microscope and also by counting transects on micrographs. The affinity ratios are shown in Table 6/2:

Table 6/2. Comparison of 3 viruses and their antisera using affinity ratios from the GLAD method of decoration.

VIRUS	COUNTS IN E.M. G/pA CONJUGATED WITH			COUNTS ON MICROGRAPHS G/pA CONJUGATED WITH		
	a.U1-TMV	a.U2-TMV	a.ToMV	a.U1-TMV	a.U2-TMV	a.ToMV
U1-TMV	—	0.3	0.3	-	-	-
U2-TMV	7.1	—	1.1	7.1	—	1.4
ToMV	16.8	1.3	—	5.1	1.2	-

The clearest identifications shown in this table involve the two closely related viruses U2-TMV and ToMV when their homologous antisera are compared with a.U1-TMV. This again confirms the closeness of the relationship between the two viruses and explains the difficulty in separating the two using just the antisera to the two of them, as can be seen from the appropriate figures in the table.

The misidentity of U1-TMV in the above experiment is difficult to explain but part of the explanation is the stronger affinity that U1-TMV has for the antisera compared to the affinity of the other two viruses for a.U1-TMV.

6.3.3. *The GLAD technique and Tymoviruses.*

All of the previous work on the GLAD method utilised rod-shaped plant viruses and it was of interest to see whether the method was applicable to isometric plant viruses. There are two main reasons for this (1) Isometric viruses appear to be less prone to fragmentation when put onto antiserum coated grids and (2) a supply of some infected plant tissue was available as well as some antiserum to them, one of which had been prepared in hens rather than rabbits.

Infected leaf tissue was available from the Virus Ecology Research Group in the Research School of Biological Sciences at the Australian National University. Three viruses were available: Turnip yellow mosaic virus (TYMV), Kennedya yellow mosaic virus (KYMV) and the Cardamine isolate of turnip yellow mosaic virus (TYMV-Cd). The antisera that were supplied were to KYMV (prepared in rabbits) and TYMV-Cd (prepared in hens). According to Koenig and Lesemann (1979) TYMV and KYMV are very closely related, only being separated by a serological differentiation index of 2.

Experimental

Initially KYMV was compared with a Tobamovirus (ToMV) by treating grids of each with G/a.KYMV and G/a.ToMV. Because these viruses are completely unrelated gold was only present in the homologous treatment, indicating that the method worked and that the antiserum to KYMV was suitable to use in the same way as previously determined for the Tobamoviruses. The gold particles generally appeared to be on the particles of KYMV.

Before doing any tests using gold on TYMV-Cd, the hen antiserum (which reportedly had a low titre) was tested for suitability in trapping and decoration. For decoration purposes both a.TYMV-Cd

and a.KYMV were used. The antiserum seemed to work satisfactorily for both purposes. It was then conjugated with G/pA for tests of the GLAD method.

Two experiments were conducted using a.TYMV-Cd. The first was a comparison of TYMV-Cd and KYMV using antisera to both conjugated to G/pA. For some reason there were almost no particles of TYMV-Cd on the grids, and no gold. Counts could be made of the KYMV grids and the 36 affinity ratios had a mean value of 1.6 but a wide range of values from 0.76 to 5.0.

The second experiment was conducted along the same lines using 3 viruses: TYMV-Cd, KYMV and the Tobamovirus ToMV. Gold was conjugated to each of the 3 antisera. Because it was an old supply of gold and only a small quantity was available, all combinations of virus and antisera were not used. Instead the experiment was set up in two halves - one was a comparison of KYMV and ToMV to check that the G/pA was still working and the other was a comparison of TYMV-Cd and ToMV. In the first half only the homologous combinations had gold particles present indicating the method was working. In the second part there was not a high density of TYMV-Cd particles present and there was no gold present with either treatment. On the ToMV grids there were about 4-8 gold particles present per binocular field on the G/a.TYMV-Cd treated grids and much higher numbers present on the G/a.ToMV treated grids. This half of the experiment was repeated at a later time with a new G/pA preparation and gave the same results.

Discussion

This series of experiments shows the GLAD method will work on isometric viruses such as the Tymoviruses, as could reasonably be expected. Problems did arise with the use of an antiserum produced

in hens rather than in rabbits, even though the antiserum worked well for particle trapping and decoration.

Even though the comparative tests showed the close relationship that exists between TYMV-Cd and KYMV, and identified the viruses correctly, the affinity ratio had a far greater spread of values than would have been expected from similar tests with Tobamoviruses. Subsequent results indicated that when an attempt is made to conjugate G/pA and a.TYMV-Cd the resulting product adheres non-specifically to grids. This may be because pA does not have a strong attachment to hen antibodies and when it is used it acts more like G/pA does when used after decoration. Such non-specific attachment could explain the wide variation seen in the experiments with TYMV-Cd and KYMV. As hen antiserum is not commonly used, at present, in serological work the point was not pursued in any further detail.

6.4. General Discussion

These experiments demonstrate that antibody labelling can be used successfully on liquid preparations on an electron microscope grid. The publication of some of the early results (Pares, Whitecross, in press) constitutes the first record of a successful attempt to label antibodies on virus particles that are present on a grid. It has now been shown that it can work on two types of plant virus groups with differing particle morphology.

When the gold labelling is applied to decorated particles the method works but is only apparent when detailed counts are made of the gold particles that are associated with the individual virus particles. This applies even when the two antisera are not related to each other, but does not happen when the GLAD modification is

used, as there is no high level of non-specific attachment over the grid surface.

If a homologous virus - antiserum combination is used with the GLAD technique a lot of the gold particles appear to be attached non-specifically to the grid surface but this proves to be not so after further investigation. There are two main bits of supporting evidence for this conclusion: (a) It does not happen when a distantly related or unrelated antiserum is used and (b) when preparations are examined at higher magnifications many of the gold particles are attached to very small pieces of the virus that can both occur in populations naturally and more of which are generated by trapping virus particles (see section 4).

This aspect of the research program has only been able to concentrate on promoting the basic concept that such a method could be developed, and demonstrating that once a method was found it could be used successfully. Having done this, it has opened up a number of possibilities for further development and refinement, and some of these are: (a) the determination of the optimum combining ratio of G/pA and antiserum; (b) development of possible modifications to obtain a greater association of gold particles with rod-shaped virus particles; (c) an investigation of very distant serological relationships between viruses, an area of work for which the GLAD method should be well suited; (d) the use of cross-absorbed antisera to distinguish closely related viruses; (e) the use of colloidal gold with different particle sizes (Frens, 1973) to distinguish between viruses with a similar appearance that occur on the same grid; (f) the use of colloidal gold conjugated to goat-anti-rabbit gamma-globulin, which is now commercially available, with different sized gold particles. An investigation of these, and other possibilities

that doubtlessly exist, should prove to be of great value in the field of plant virus identification.

Labelling antibody particles by the GLAD technique has some advantages over the standard decoration method in the identification of viruses on an electron microscope grid, and these are:

(1) The GLAD method gives a better, unequivocal, identification of the presence of antibodies as has been found when gold has been used to label antibodies attached to antigenic sites in sectioned material.

(2) The GLAD method enables very distant relationships to be determined in a situation in which decoration would be too light to be detected or determined with any reliability.

(3) The GLAD method offers a greater economy of antiserum than decoration in normal circumstances as the gold/protein-A can be used with antiserum at a dilution of 1:1,000.

(4) The GLAD method, in most routine diagnostic situations, obviates any necessity to do preliminary tests with dilution series of antisera to find a suitable diagnostic concentration for each set of viruses being examined.

(5) The GLAD method, as with conventional decoration, has an advantage over other serological methods in that, in theory, only one virus particle is necessary to get a result. In practice diagnosis can be made on very low numbers of virus particles, too few to give any result by conventional serology.

7. APPLICATION OF IEM TO FIELD SURVEYING OF VIRUS INCIDENCE

"Life is the art of drawing sufficient conclusions from insufficient evidence."

Butler.

7.1 Introduction

Viruses are frequently detected in samples from cultivated crops or wild plants by electron microscopy of negatively stained sap extracts. The reasons for this are that the technique is quick and virus particle morphology can often place the virus into a particular group. This applies principally to the rod-shaped plant viruses which have a sufficient range of sizes and appearance to allow their grouping to be recognised, and this has the additional advantage that it allows one to predict a range of their other characteristics. The development of the use of serum-coated grids for extracting viruses from infected sap has opened the way for detection of isometric plant viruses. Unfortunately they do not have such a diversity of shape and size to allow unequivocal grouping based on morphology of the particles. There is thus more need for some method of unequivocally identifying isometric plant viruses by electron microscopy.

When rod-shaped viruses are considered, there is sometimes (but not always) a need for a more accurate diagnosis than merely allocating the virus to a group. Usually such a diagnostic procedure relies on time-consuming tests such as host-range studies, serological tests, etc. Considerable savings of time could be made if identification could be done in the electron microscope using sap extracts.

IEM would seem to be the ideal way by which to achieve this and in the previous sections I have examined the features that can be used for identification, the factors that can affect them, and the possibilities for quantifying the method to enable identification of the viruses. It is now appropriate to see how these methods work in a situation in which one needs to identify viruses occurring in a crop. It is important when doing this to use crude sap preparations rather than ones that have been partially purified as this is where

the real value of the IEM technique lies.

The two crops that I selected were orchids and tomatoes, both of which are commonly infected by Tobamoviruses. In New South Wales the exact identity of the viruses is not known, but should be, and in both of these plant types there is more than one Tobamovirus capable of infection.

Throughout the world the most common Tobamovirus in tomatoes is ToMV, and other viruses from this group are not found often as most of them compete poorly in tomato and some (e.g. U2-TMV) infect tomato with difficulty or not at all (Hollings and Huttinga, 1976).

Some of the questions that need answering, at least as far as the State of New South Wales is concerned, are (1) What is the most common Tobamovirus infecting tomatoes? (2) How do the Tobamoviruses present in tomatoes compare with ToMV and U1-TMV? (3) If more than one Tobamovirus is present, how prevalent is each type?

Tobamovirus infections of tomato in N.S.W. are widespread in glasshouse crops and I only sampled crops from the Sydney area.

The situation of Tobamoviruses in orchids is less clear. Kado et al. (1968) made a detailed study of 7 isolates from orchid and found that they differed from type U1-TMV. While acknowledging that ORSV had been recorded previously by other workers, they considered it to be a strain of U1-TMV, and they did not compare any of their strains with ORSV. Since then it has been shown that ORSV is sufficiently distinct from U1-TMV to be considered a separate Tobamovirus, distantly serologically related to U1-TMV, and the information on ORSV has been summarised by Paul (1975). It does seem as if the

main Tobamovirus in orchids is ORSV but that there may be some infection with U1-TMV.

Previously I have examined samples of orchids in N.S.W. and found that they commonly contain a Tobamovirus that is characterised in negatively stained sap preparations by having a high proportion of particles less than 300 nm long. This is more like a population of ORSV particles than one of U1-TMV. No work has been done, other than particle morphology studies, in N.S.W. to determine the identity of the virus.

The following experiments were set up to examine the Tobamoviruses in both hosts by IEM to obtain some identification of the viruses by comparative particle counts, decoration and GLAD. The purpose was two-fold, one to obtain information about the viruses and secondly to evaluate the various aspects of the use of IEM in a situation in which the infections are not being controlled experimentally.

7.2. Orchid Tobamoviruses

At the start of the project there was no virus isolate or antiserum of type ORSV to use and so one was selected from those surveyed. As stated in the Materials and Methods (section 2) it appeared from particle morphology and host reactions to be the same as ORSV. It was used throughout all the experimental work that has been done and has behaved as expected of type ORSV. Conversely the results have consistently shown that this isolate from orchids is not closely related to U1-TMV, U2-TMV or ToMV.

Some other experimental work, including a limited survey of the incidence of this group of viruses in orchids, has been done on some of the isolates and will be reported here.

7.2.1. *Field Survey.*

Orchid leaves were sampled from 38 plants growing in the Royal Botanic Gardens, Sydney; Yarralumla Nursery, Canberra and various houses in the Sydney area.

Each sample was treated by removing a small piece of the leaf tissue (ca. 4 x 4 mm), macerating it in 2% ammonium molybdate and putting a drop of this onto an electron microscope grid, drying it and then examining it in the electron microscope for the presence or absence of Tobamovirus-like particles. The genera examined are given in the following list followed by the number having Tobamovirus particles/number sampled.

Laeliocattleya	6/11	Cattleya	2/5
Dendrobium	0/4	Laelia	0/1
Phalaenopsis	2/5	Vanda	0/2
Peristeria	0/1	Spathoglottis	1/1
Vandopsis	1/1	Cymbidium	2/7

Out of the 38 samples, 32 (84%) were infected by either a Tobamovirus, a Potexvirus or a small bacilliform virus. Tobamovirus particles were present in 14 (37%) of samples.

All of the samples showing particles like those of a Tobamovirus were inoculated onto Nicotiana tabacum cvs. Samsun and White Burley and Chenopodium quinoa. The appearance of the particles and the reactions of the indicator hosts indicated that the plants were all infected with a Tobamovirus that was most likely to be ORSV.

The results indicate that quite a high percentage of orchid plants are infected by a virus and some plants have a double infection. The general infection level would be expected to be much lower in a commercial enterprise where a great deal more care is taken to prevent infection and subsequent spread.

Doing the survey of orchids with untrapped preparations showed up the deficiencies compared to using trapped preparations. One of these was that the virus particles were in relatively low numbers and the grids had to be examined for a considerable time to detect the virus particles. This applied particularly to the small bacilliform particles. It may well have been that some of the samples that appeared to be negative may have been infected but the particles were in too low a concentration to be readily detected.

Another shortcoming of this method is the fact that the only thing that can be said with any certainty is that a Tobamovirus is present and from the particle morphology is more likely to be ORSV than U1-TMV. To gain further information indicator hosts would have to be inoculated (and it could take about 2 weeks for symptoms to develop) or some type of serological test would have to be set up.

7.2.2. *Immuno-electron microscopy.*

When the orchids were surveyed they were numbered E1, E2 ... in the order in which they were sampled. Five of these were selected for limited further study and these were:

E27 - Cattleya - infected with a Potexvirus.
E28 - Cattleya - infected with a Potexvirus.
E29 - Phalaenopsis - infected with a Potexvirus + a Tobamovirus.
E30 - Cattleya - infected with a Tobamovirus.
E37 - Cymbidium - infected with a small bacilliform virus.

Experimental

Each of the samples was trapped onto 2 grids, one coated with a.U1-TMV and one with a.ORSV diluted 1:1,000. The grids were then floated on drops of leaf tissue macerated in PB overnight at 5°C, and negatively stained before examination. There were no

particles present on either antiserum with samples E27, E28 and E37. Samples E29 and E30 had Tobamovirus particles present on both antisera in much higher numbers than were seen in the original survey, but there were many more particles present on the a.ORSV grids and this was very obvious without any need to count the particles. The numbers of particles on the E30 grids were greater than on the E29 grids.

The two positive samples were then trapped onto grids as above to verify the relative particle numbers. Four extra grids of a.ORSV-E30 were prepared and then treated with a.U1-TMV, a.ORSV, G/a.U1-TMV and G/a.ORSV. At all stages a dilution of 1:1,000 was used for the antisera. The virus particles were present in the same proportions as seen in the first set of trapped grids. As expected the decorating antisera were both too dilute to show any evidence of decoration. The means of 10 counts of the gold particles on each grid were G/a.ORSV-23.1; G/a.U1-TMV-5.1, a ratio of 4.5:1.

Discussion

Comparisons of particle numbers are useful for comparing viruses that are as distantly related as U1-TMV and ORSV as it does not involve any necessity to do detailed particle counts. There are 3 possible explanations for the fact that there were fewer particles present on the E29 grids than on the E30 grids:

1) It is due to the fact that the 2 orchid genera involved support different concentrations of the same virus in a fully developed infection.

2) E29 and E30 are the same virus but were at different stages of the infection process when sampled.

3) E29 and E30 are two different strains of ORSV and that E30 is more closely related to the isolate used to produce the antisera.

The answer to the explanation lies in further detailed experimental work that would have to be done to look at the variations in a wide variety of isolates of ORSV in orchids in N.S.W.

The use of GLAD shows quickly and simply that isolate E30 is more closely related to ORSV than to U1-TMV, both by counting the gold particles and by visual assessment of the grids.

The information obtained using a.ORSV in earlier investigations of the IEM method also confirms these findings.

Using various aspects of IEM, the results show a considerable uniformity and demonstrate that the Tobamoviruses from samples E29 and E30 are not U1-TMV, but have a distant relationship to it and are most probably strains of ORSV. Because of their behaviour in indicator hosts, and their particle morphology it is also most likely that the other 12 isolates of Tobamoviruses were also strains of ORSV rather than U1-TMV, and would be in agreement with the findings of Kado et al. (1968) and Paul (1975).

There were two points to doing these experiments, one of which was to find information on the virus in orchids. The other was to evaluate the methods. In general the latter have proved very useful and much simpler and quicker than most of the more conventional methods.

The fact that the original survey was done by using untrapped preparations and subsequently checked by IEM does show up one disadvantage of IEM. This is that, when an untrapped preparation is examined, the virus density is low and allows the detection of any virus that may be present. When IEM is used and a virus related to the antiserum coating is present, the particles are present in such a density that particles of another virus are obscured. Compounding

this is the fact that the IEM method is much less sensitive in detecting viruses in a preparation that has no particles related to the antiserum coating, than the technique of negative staining used in the survey of orchid viruses.

7.3. Tomato Tobamoviruses

7.3.1. *Survey of glass house crops.*

Glasshouse tomato crops from 6 growers in the Warriewood, Mona Vale and Ingleside areas near Sydney were sampled. Assistance from local horticulturists of the N.S.W. Department of Agriculture is gratefully acknowledged, as is help given in sample collection and examination - under my supervision - by technical staff of the Biological and Chemical Research Institute, Rydalmere, N.S.W.

Large shoots were removed from each of the 71 plants selected. Some plants were showing no symptoms of infection but the majority were showing some symptoms of a virus infection. After collection the samples were brought to the laboratory and a few leaves were removed from each shoot and macerated, 1 g per 3 ml 0.06 M PB. The macerate and the remaining shoot samples were then stored frozen until used for preparation of grids.

Grids were prepared by trapping the macerate from each sample for 3 hours onto one grid coated with a.U1-TMV and one coated with a.ToMV. The grids were then washed and negatively stained before examination. Because of the large numbers of samples involved, because the technique was being evaluated in its applicability, and because of the generally high numbers of virus particles, the grids were estimated visually without counts and the density of virus particles was rated on a scale of 1 (low density) to 5 (high density). Based on this ranking

system the samples could be put into 4 categories: (1) positive U1-TMV; (2) positive ToMV; (3) not known - usually because particle numbers were too low to detect a difference. In a few instances many particles were present but separation difficult; (4) Negative.

Table 7/1 shows the varieties examined, the number of each and the number in each of the above 4 categories.

Table 7/1.

VARIETY	NO.	NO. IDENTIFIED AS: U1-TMV	ToMV	NOT KNOWN	NEGATIVE
GROSSE LISSE	40	2	26	10	2
MANAPAL	30	3	21	6	0
PRECADOR	1	0	0	1	0
		7%	66%	24%	3%

This shows that a very high percentage of the sampled plants were infected by a Tobamovirus and the majority appeared to be definitely infected with a virus that was much more closely related to ToMV than to U1-TMV. Of the 52 plants found to be positive for one or other of the viruses, 20 of them were such that the rating on one antiserum was within 1 rating point of the other antiserum. Doing such a quantitative estimate by electron microscopy is always difficult and these results help to reinforce this fact.

Doing the survey in this manner does give a better idea than untrapped preparations do of the identity of the viruses involved, provided the serological relationship is distant enough, as is the case with the two viruses that are possibly infecting tomatoes. In contrast to the orchid survey it does not give any idea of any other viruses that may be infecting the plants.

Out of the samples that yielded an apparent difference in particle numbers, about 90% had ToMV present, which is in keeping with the fact that U1-TMV competes poorly in tomatoes. In addition, of the 5 that apparently had U1-TMV present, 4 had very low particle numbers, making evaluation difficult without counts and the other had a reasonable number of particles present but little difference between the two grids. In these instances it is quite possible that counts could have shown the virus to be closer to ToMV.

Again the survey shows that many of the plants had a very low particle density, as happened with some of the orchid samples. The incidence of this in tomatoes was about 25%, and this could be due to a variation in the strain present or a variation in the stage of the infection.

7.3.2. *IEM examination of 5 samples.*

For further study a selection was made of 5 of the samples that had varying characteristics, based on the visual estimates of particle density, and these were as follows, as well as the ratings given for each of the two antisera:

Sample Nos:	5	22	42	69A	69B
a.U1-TMV :	+	+	++	++	+++
a.ToMV :	+	++	+	++	++++

These 5 samples were then tested by particle trapping, decoration and GLAD.

Experimental

Particle numbers were compared by trapping each of the isolates onto 2 grids, overnight at 5°C. One grid was coated with a.U1-TMV and the other with a.ToMV. The mean of 10 counts from random fields on each grid was calculated.

The antiserum dilution that was used for diagnostic decoration was determined by taking one isolate (69A), that was not differentiated in the original survey, and then decorating with a range of dilutions of a.U1-TMV and a.ToMV. The dilutions tested were a two-fold series from 1:16 - 1:512, and the decoration was done for 15 minutes. The grids were each examined for evidence of the distinct dark halo described and illustrated by Milne and Luisoni (1975), and the results were:

Dilution	1:16	1:32	1:64	1:128	1:256	1:512
a.U1-TMV	+	?	-	-	-	-
a.ToMV	+	+	+	+	?	?

Based on these results a dilution of 1:64 was selected and the 5 isolates were decorated with each of the antisera for 15 minutes.

The GLAD method was then tested on these isolates using G/a.ToMV and G/a.U1-TMV as described in section 6. Counts were made on 10 random fields per grid and the means of the counts used for comparison. The experiment was also repeated with ToMV included.

The results of this series of experiments are shown in the following Table 7/2.

Table 7/2. Comparison of 3 IEM methods to determine the relationship of 5 isolates of Tobamoviruses from tomato, U1-TMV and ToMV. The figures are affinity ratios*using a.ToMV as the homologous antiserum. The decoration was classified as heavy (++), light (+) or undetectable (-).

	VIRUS PREPARATION						
	5	22	42	69A	69B	U1-TMV	ToMV
VIRUS COUNTS :	4.6	15.6	7.5	7.5	6.9	NT	NT
GLAD -- (1) :	1.3	4.4	4.4	2.6	6.7	NT	NT
GLAD -- (2) :	2.2	4.0	3.1	2.3	4.2	NT	2.5
Decoration - a.U1-TMV	+	-	-	+	-	++	-
- a.ToMV	++	++	++	++	++	+	+

NT = Not tested.

* see page 44

7.3.3. *Comparison of isolates 69A, 69B.*

Based on the field survey and the subsequent studies on 5 of these isolates, 2 isolates were then subjected to further experimentation. One of these (69A) was present in relative low numbers in the sap exudates and appeared to be slightly more closely related to U1-TMV than the other (69B) which appeared very closely related to ToMV.

Experimental

The previous differences seen between the two isolates were first confirmed by trapping each of them overnight on two grids, one coated with a.U1-TMV and one with a.ToMV. Counts were not necessary with isolate 69B as there were very high numbers of particles present with a.ToMV (almost too dense to be counted) and obviously far fewer present with a.U1-TMV. There were very low numbers present on both antisera with 69A, and the mean of 10 counts for each were:
a.U1-TMV : 2.7, a.ToMV : 3.3. This is very similar to the situation seen in the original survey.

The two isolates were then compared with U1-TMV, U2-TMV and ToMV by trapping them each onto 3 grids, one coated with each of the antisera of the 3 viruses. In order to reduce the particle numbers, and possibly maximise any differences that may exist, the grids were pre-treated with protein-A and the trapping antisera used at 1:1,000. The results were as shown in the following Table 7/3.

Table 7/3. Comparison of tomato isolates 69A and 69B with U1-TMV, U2-TMV and ToMV by comparing numbers of virus particles expressed as affinity ratios.

ANTISERUM	VIRUS				
	U1-TMV	U2-TMV	ToMV	69A(a)	69B
a.ToMV	1.0	1.0	1.0	1.0	1.0
a.U2-TMV	0.9	0.8	1.2	1.1	0.7
a.U1-TMV	1.0	1.3	4.4	9.0	4.5

(a) Particle numbers very low, the mean no. per field for a.ToMV being only 1.8.

Isolates such as 69A could be present on grids in low numbers because it was in a low concentration in the plant or because it was a different virus that was distantly related to both U1-TMV and ToMV. There is one Tobamovirus that does occasionally infect tomatoes and this is Ribgrass mosaic virus (Oshima and Harrison, 1975). Some antiserum to this virus was kindly made available by Dr A.J. Gibbs, Research School of Biological Sciences, Australian National University. The two isolates 69A and 69B were trapped onto 1 grid coated with each of the antisera to U1-TMV, U2-TMV, ToMV and Ribgrass mosaic virus. Although the results for the first three antisera were similar to those in the table above, there were virtually no particles present on the grids coated with the antiserum to Ribgrass mosaic virus.

While the above experiments were being done the two isolates were inoculated onto tomato seedlings in the glasshouse and left until symptoms were showing on most of the leaves. These plants were then sampled and sap exudates tested by trapping virus overnight and examining the grids to determine the number of particles of each trapped by a.U1-TMV and a.ToMV. In both isolates the number of particles on the grids that had been coated with a.ToMV were extremely high and virtually uncountable, while there were obviously many less on the a.U1-TMV grids.

Additionally three experiments were set up to examine the two viruses by using the GLAD method with G/a.U1-TMV and G/a.ToMV. In one experiment both viruses were tested at the same time and in the other two each of the two isolates was tested separately. The test with the two isolates was done using the samples that had been taken from inoculated tomatoes with well developed symptoms. Counts were made of the gold particles on 10 random fields, and confirmed the findings reported in 7.3.2.

7.3.4. *Tests on isolate number 5.*

Using antiserum to U1-TMV and ToMV, isolate 5 was examined by both particle numbers and the GLAD technique.

The particle trapping comparisons were done using isolate 5 alone with acquisition times of 15 minutes and 3 hours and also in comparison with isolates 22 and 42 with trapping times of 3 hours and overnight. The affinity ratios were:

ISOLATE	:	5	22	42
3 hour VAT	:	6.1	68	12.2
overnight VAT	:	10.7	9.9	15.4

When isolate 5 was compared with the two antisera the figures were 2.2 for the 15 minute acquisition and 48 for the 3 hour trapping.

In the tests using the GLAD method isolate 5 was compared alone with the two antisera giving an affinity ratio of 1.9. It was also compared with isolates 22 and 69B, the figures being 3.8, 7.9 and 8.0 respectively.

7.3.5. *Discussion.*

This section will deal primarily with the identity of the isolates from tomatoes. The relative usefulness of IEM in determining the identity of field isolates will be left for section 7.4 and discussed in conjunction with the results from orchids.

Although the original tomato samples contained 7 samples selected as symptomless, only one of these showed up as negative, 3 of them had a very low virus concentration and could not be diagnosed and 3 had reasonable numbers of virus particles present and appeared to be infected with tomato mosaic virus.

Of the 69 samples infected with a Tobamovirus, and that were diagnosed as one or other of the viruses, about 10% were apparently infected with U1-TMV. This is in agreement with the idea that viruses other than ToMV compete poorly in tomato, but is perhaps somewhat higher than might have been expected. Sample 42 had a relatively low virus concentration and appeared to be infected with U1-TMV, was fairly representative of that group, and was therefore selected for further studies.

Also selected for further study was No. 22 that had about the same virus concentration as No. 42 but had slightly more particles estimated on the grid coated with a.ToMV. Further quantitative estimation by virus particle counts, decoration and GLAD showed that both these isolates were very similar to each other and much more closely related to ToMV than to U1-TMV.

Two other viruses (5 and 69A) examined in more detail were originally present in low numbers and could not be diagnosed as being closer to one or other of the antisera by estimation. One of these (5) was present in much lower concentration than the other. Because of

the low virus numbers, and lack of greater numbers being estimated on one or other antisera, it was considered a possibility that these two (and others like them in the survey) may have been a different virus somewhat distantly related to both U1-TMV and ToMV. This would have accounted for the low numbers and lack of differentiation. So also would a very low concentration of one of the two viruses and the fact that counts were not done in the survey. Subsequent testing by various IEM methods indicated that the two isolates are both more closely related to ToMV than to U1-TMV, but are different to the other 3 isolates (22, 42, 69B) and have more cross-reactivity to the anti-serum against U1-TMV. This is more clearly indicated by GLAD and decoration than by virus particle counts.

The fifth isolate selected for the additional study was 69B, that had a high number of particles present and was obviously more closely related to ToMV, even when the grids were estimated rather than counted. All subsequent tests indicated that this isolate behaved in a very similar fashion to 22 and 42 but was perhaps slightly more closely related to ToMV than either of these.

It would seem from all of the results that the 5 isolates studied are all closer to ToMV than to U1-TMV, and they can possibly be divided into 2 groups, one of which (Nos. 5 and 69A) has more cross-reactivity with a.U1-TMV than does the other group (22, 42, 69B). There are some indications that 69B may be even closer to ToMV than 22 and 42, and so there may possibly be 3 groups in the 5 isolates.

7.4. General Discussion

The two field surveys were carried out in different ways, that on orchids being done by simply negatively staining expressed sap,

and the one on tomatoes by trapping particles onto grids coated with antisera. The method used for orchids had the advantages of being quicker and simpler to do and allowing detection of a range of viruses that may be present. When the samples are trapped onto grids coated with different antisera it has the advantages of giving some idea of the identity of the virus in many instances and also enabling the detection of infections in which the virus is present in a very low concentration and which would be very unlikely to be detected in untrapped preparations.

If it is desirable to do a survey in which it is necessary to detect a range of viruses in different virus groups this could be overcome by coating grids with a mixture of the antisera to each of the viruses of interest. This would then combine the advantage of negatively staining sap with that of IEM and enable detection of a range of viruses even if one or all were present in very low concentrations. There is the possibility that if one virus were present in a very high concentration in the plant (e.g. ToMV in tomato) then the density of this virus may obscure the particles of another virus that was present in a low concentration. The use of such "multiple trapping" would not differentiate between two viruses belonging to the same group (e.g. U1-TMV and ToMV), but this could be done later by decoration or GLAD.

Though trapping samples on grids coated with antiserum does offer the possibility of diagnosis, the conditions have to be chosen carefully. In the survey of tomatoes I thought that a virus acquisition time of 3 hours would be sufficient because Tobamoviruses usually are present in such high concentrations in infected tissue. In general this was the case but in many samples the virus particles

were present in very low numbers on the grids and diagnosis was not possible. When some of these were subsequently re-tested with an acquisition time of about 18 hours, there were many more particles present on both grids and a diagnosis could be made. A long acquisition time is not the complete answer as, when this was tested on samples that had a high concentration of virus present, the number of particles on the heterologous grid increased to such an extent that estimation of relative numbers was impossible and the virus density on both grids was such that counting also became impossible.

Visual estimation of relative particle numbers can be used to successfully diagnose some of the samples but it has been shown that when there is only a small difference in the numbers on two grids the estimate can be wrong (e.g. sample 42). When the original ratings of the tomato samples are examined there were only 29 in which there was a substantial difference in the ratings for each grid. Of these 28 were diagnosed as closer to ToMV and 1 as closer to U1-TMV and this one sample had such low numbers present there were none seen on the grid coated with a.ToMV and only a few on the a.U1-TMV grid.

This means that there were only 28 samples that could be diagnosed with reasonable certainty and 43 (61%) could not. The question then is how best can these samples be diagnosed using IEM as the basis for diagnosis? The three approaches using IEM are (1) counting particle numbers, (3) Decoration, and (3) the GLAD method.

With field samples, particle counting can have the disadvantage of depending greatly on the virus concentration in the sample and vary from so few that counting shows no difference to so many that counting is impossible. If the particular virus was one that did not reach a very high concentration in the host then the

latter would not be a problem. Even with the one sample the proportion of the counts with 2 antisera can vary greatly. This can be illustrated by samples 5 and 69A in which the affinity ratios, using a.ToMV as the homologous antiserum, varied from 2.2 - 48 in the case of isolate 5, with a mean of 16.8 ± 10.5. The corresponding figures for 69A being 1.2 - 9 with a mean of 4.3 ± 2.4. All of these figures indicated a relationship closer to ToMV than to U1-TMV but indicated a distant relationship to U1-TMV.

Examination of the same two isolates by decoration indicated a somewhat closer relationship to U1-TMV than did particle counts and this is confirmed by the GLAD tests. All tests indicated that the viruses were probably strains of ToMV. Both decoration and GLAD were more consistent in their results as they do not depend on the initial concentration in the host plant. Thus for most diagnostic purposes it would seem that decoration or GLAD are preferrable to particle counts. If the same ratios as used above in particle counts are compared for the GLAD counts they had a range of 1.3 - 3.8 for isolate 5 with a mean of 2.3 ± 0.5. The figures for 69A were 1.0 - 2.6 and a mean of 1.9 ± 0.4.

Decoration has the disadvantage of light decoration being hard to identify with certainty and any evaluation of light and heavy decoration being subjective in the same way as estimating relative particle densities. Additionally decoration would not detect very distant relationships as well as would GLAD.

This all raises the point as to what approach would be best for detection and diagnosis from field samples.

There are essentially two types of situation in which field samples could be examined. One is that of a limited number of

samples being taken from a crop and the other is that of a wider-scale survey being undertaken, such as has been described in 7.3 for tomatoes. I consider that each of these would necessitate a different approach, mainly from the point of view of the volume of work that would have to be done, and the ability to get it done in a reasonable time interval. Where only a few samples from a crop are concerned they could be trapped onto grids coated with different antisera and detailed counts could be done on each. This approach would make the task impracticably large in a survey with 100 or more samples. In fact the small-sample situation presents few problems as a range of variations are possible for each method. The real problem arises in the large-scale survey and this will now be considered.

It is realised that, in such a large-scale survey, for any given crop or host plant there are many permutations and combinations of the number of possible related viruses that could be present and the varying serological relationships that may exist between them. Appropriate modifications would, of course, have to be made in each instance. For the purposes of this discussion and to elucidate some principles of the method of survey I will use the model of tomato plants and the two viruses U1-TMV and ToMV as the most likely ones to be present, and for which the survey is to be done.

The survey reported here has shown that trapping each sample onto 2 grids and estimating relative particle densities seems only to give a positive result in about 39% of the samples. Doing counts on all of the samples or even the remaining 61% is a very long job and in many instances the virus density was too low or too high to obtain a result. It would be more efficient to be independent of the variation in virus content. To do this the samples would be best trapped onto a single grid coated with a mixture of the two

antisera for each sample. The trapping time should be sufficiently long to ensure that viruses could be trapped from those samples with a very low concentration present. In the case of the two viruses in tomato this time would seem to be not less than 3 hours. For other viruses in other crops the time may very well have to be at least overnight (e.g. Luteoviruses).

Each grid should then be treated for 10-15 minutes with antiserum at a suitable dilution determined by previous tests. In the case of tomatoes this would be antiserum to ToMV at 1:64. The particular antiserum is chosen because most samples would be expected to be ToMV or a strain of it and this would give positive decoration of most samples. An examination of the grids should separate most of the samples accurately and in tomatoes most samples would probably be positive. There would be some samples showing negative and some showing only light decoration. These would then be tested in more detail by the GLAD method.

Such a series of tests would separate any samples infected with U1-TMV from those infected with ToMV, but would not pick up different strains of ToMV. In many instances this would be sufficient diagnosis but a finer diagnosis of strains would depend on a series of detailed steps of decoration and GLAD using different dilutions of the antisera. The use of GLAD on the negative samples would enable the detection of distant relationships not readily detected by the decoration method.

Using a schedule such as that suggested it would make it possible to undertake a large-scale survey efficiently and quickly. It features the more reliable diagnostic aspects of the IEM rather than the less reliable method of particle counts. The scheme becomes

independent of the variations in virus concentration in the samples in almost all cases except that of extremely low concentration. In such an instance the grids showing no virus particles could be re-tested using much longer virus acquisition times to confirm the presence or absence of virus particles.

8. OVERALL DISCUSSION

"The aim of science is to seek the simplest explanation of complex facts."

A.N. Whitehead.

- *What is the best IEM method to use for plant viruses?*
- *Can IEM be used to increase plant virus particle numbers for size distribution determination?*
- *What Tobamoviruses infect orchids and tomatoes in N.S.W.?*

These are three key questions which the preceding sections supply some of the answers that, in turn give an understanding of this new technique, how it can be manipulated for a particular purpose and its advantages, disadvantages and limitations.

The realisation by Derrick (1973b) that grids could be coated with antiserum and then used to increase the density of virus particles on a grid heralded the start of an extremely valuable and sensitive tool for virus detection and identification. In the introduction I have shown that it has since been subjected to many minor and major variations, modifications and additions. It has proved to be more sensitive than any other method so far developed but the concept and development is still in the early stages. In spite of the fact that there has been widespread use of the basic method and the modifications, my research program, as detailed in sections 3-7, represents the first comprehensive comparison of the various modifications and some of the factors that are involved in its successful use. My work also offers a new method that has proved advantageous in virus identification and with further development should prove even more so.

Any method of virus detection and identification has its principal use in the diagnosis and study of virus diseases that are economically important in commercial crops, and this includes the incidence of various viruses in natural plant communities that act as reservoirs, or potential reservoirs, for the infection of commercial

crop plants. Because of this any new method must be of use in such a situation as well as in laboratory conditions. Any new method also has to offer some advantages over the range of methods that were used prior to its development.

In this discussion I will briefly look at the "normal" methods used in plant virology, i.e. those that were used prior to IEM, and then examine the various aspects of my results in relation to the points mentioned above. I do not propose to discuss the various sections of my work in detail as this has been done in each of the appropriate sections.

8.1. The "normal" method of detection and identification of viruses from infected plant tissues

As a first step, samples are examined by crushing infected leaf tissue in negative stain and examining the resulting preparation in the electron microscope. For most rod-shaped and bacilliform viruses this method is satisfactory, and in the case of rod-shaped viruses can usually result in their being placed as members of a certain virus group (e.g. Tobamovirus, Potyvirus etc.). Isometric viruses are often hard to detect because of the difficulty in differentiating them from plant sap constituents, and even if they can be detected they do not vary sufficiently in morphology to allow a grouping. Any virus in a very low concentration is unlikely to be detected by this method.

If more accurate diagnosis is needed, or an isometric virus is to be detected, there is then need to resort to a range of other tests such as serology (tube precipitin, microprecipitin, gel diffusion, etc.) or host range studies. As with any method they have their disadvantages. Serological methods often require some concentration

of the virus sample from a relatively large amount of infected leaf tissue, and are less economical of time and reagents than IEM. Host range studies can take long periods of time and glasshouse space. Some host studies take 8 days to produce symptoms and in the case of fruit tree viruses can take years. Some viruses are not mechanically transmissible and virus-free colonies of the insect vector (if it is known) are needed. There is thus clearly a need for improvement over these standard methods, and the data presented in the early sections show that IEM can overcome most of these disadvantages.

8.2. The IEM method

All techniques based on serology suffer from the disadvantage that some idea of the virus or virus-group involved is necessary before the tests can be done. If there is no idea of the virus then the possibilities of which antisera to use and at which dilutions they are to be used may be too great to enable the tests to be done. The technique of IEM, while no less subject to this disadvantage, does offer the possibility of coating the grids with more than one antiserum, one to each of the viruses that are known to infect the possible host. If the possible viruses belong to virus groups that have different morphology, the viruses may then be identified. With no other serologically based technique can this be done as easily.

The use of IEM can be divided into 2 main areas (1) virus detection and (2) virus identification, and it is proposed to look at the various aspects of the preceding experimental work in relation to these 2 uses.

8.2.1. *Virus detection by IEM.*

The two field-surveys (section 7) illustrate clearly the advantages and disadvantages of the two ways in which the initial

electron microscopy can be done. There is no doubt from the results in sections 3 and 7 that IEM, whatever variation is used, greatly improves the sensitivity of detection of a particular virus or viruses related to the antisera used to coat the grids. The penalty paid for the greater sensitivity is that other viruses are more difficult to detect due to the high density of particles related to the antiserum. When the orchid survey was done using "normal" negative staining, some samples had particles of a Potexvirus or a small bacilliform virus. Detection would be unlikely using IEM on grids coated only with a.ORSV but could be overcome by coating grids with a mixture of antisera related to each of these two viruses as well as that to ORSV.

When using IEM to detect a particular virus or viruses, the method can be modified in a variety of ways to suit the system being studied at that time, and this should be done. This modification is not as easily done when detecting viruses in a range of samples in a field situation as some samples can be from plants that are in early stages of infection and therefore have very low particle numbers present. In such circumstances the method used must be designed so that maximum sensitivity is obtained. When IEM is used for the detection of virus infections (as opposed to being used diagnostically) there are only two steps in the process capable of any manipulation to give maximum sensitivity: (1) Coating the grid with antiserum and (2) virus acquisition.

Antiserum coating. Pre-coating grids with protein-A resulted in particles being trapped in greater numbers than they were with the rapid methods investigated by Shukla and Gough (1979). However since that time current usage of the rapid methods indicates that it has been realised that antiserum must be used at a dilution of ca. 1:1,000 and I have shown in section 3 that when this is done

the use of pA has no advantage over the rapid methods and does have a number of disadvantages. For this reason it does not seem to have received much acceptance as a general technique.

There is little scope for manipulation of the antiserum dilution or coating time as I have demonstrated in sections 3 and 5. It has been apparent throughout my work that the antiserum is not very efficient when used at concentrations greater than 1:1,000. Other reports in the literature on work done with viruses in groups other than Tobamoviruses tend to confirm this, and to suggest that the inefficiency of concentrated antisera is most probably due to the presence of a competitive inhibitor. Although the identity of the inhibitor is not known there is a strong suggestion that it may be partly or wholly the serum albumins, though other serum proteins may also be implicated (see 3.7). Even at a dilution of 1:1,000, a dilution that appears to work well regardless of the antiserum titre, there must be large numbers of IgG molecules present as virus particles can still be trapped in high numbers at a range of dilutions. In my work I have shown a decrease in particle numbers of only 36% when the antiserum is diluted from 1:1,000 - 1:64,000, and there has been a report of an antiserum still trapping particles at a dilution of ca. 1:3,000,000 (Derrick and Brlansky, 1976).

This all indicates that the antiserum dilution cannot be altered to maximise the detection of particles beyond the fact that it must be used at a dilution of ca. 1,000 to overcome competitive inhibitors present in concentrated antiserum. It would, however, be advisable to check any new antiserum to determine the best dilution to use for IEM. Because IEM uses so little antiserum to coat a grid (in the region of 5-10 µl), testing a range of dilutions would still

not use very much antiserum if only a small amount were available.

I have demonstrated that in all the IEM methods reported there is a wide variation in antiserum coating times, and in all the methods there has been very little comment as to why a particular time was chosen. In 3.7 I put forward an argument that there would be an increase in the amount of IgG attached to a grid as the coating time was increased and this seemed to be supported by the results shown in Table 3/2. It has not been possible to repeat this type of result and evidence presented in Tables 5/2 and 5/3 establish that increased coating time does not result in any increase in the number of trapped virus particles, and that this type of result was reproducible. I consider that the figure of 52.2 in Table 3/2 was a result of the inexplicably large or small counts that occasionally occur when particle counting is used (see 5.7).

I do think that the argument put forward in 3.7 is correct in that the amount of IgG on a grid would increase with application time, but not at a concentration of 1:1,000 at which there are sufficient IgG molecules to coat the grid within the first 10 minutes of application. I suspect that with very dilute antisera there would be an increase in attached IgG with time. As there is no practical advantage in using very dilute antisera for longer times there is, for most applications, no possibility of increasing the sensitivity of virus detection by using longer coating times. The only situations in which longer times may have to be used would be (1) with a very poor antiserum with a low titre so that there would be a low concentration of IgG at 1:1,000 and (2) with cross-absorbed antisera where the cross-absorbtion has removed a high percentage of IgG resulting in a very low concentration of IgG molecules at a dilution of 1:1,000.

Virus acquisition. In spite of claims that the virus acquisition time only results in relatively small increases in virus particle numbers with increasing time (see 3.7 and 5.3.3), I have established that the VAT is the most important step in maximising the sensitivity of detection, as shown in Table 5/3. The apparent discrepancy between reports in the literature and my findings are easily explained by the fact that virus concentration in a sample and virus acquisition time are closely inter-related as I have pointed out in 5.3.2 and 5.7. With higher virus concentrations there is less effect of increased VAT on the number of trapped virus particles, and in the published work the viruses were present at relatively high concentrations and my work was done with much lower virus concentrations.

When IEM is used to detect the presence or absence of a virus the interaction of virus concentration and acquisition time is less important than when the particle numbers are being used for virus identification, as shown in 7.3 and as will be discussed in 8.2.2. The important fact for virus detection is that the virus acquisition step be long enough to account for the fact that there may be a very low concentration in the sample, and this would necessitate a long (e.g. overnight) trapping time. When this is done there would be very high, usually uncountable, numbers of virus particles on grids from samples with a high concentration of virus present in them. This does not matter for detection purposes.

8.2.2. *Virus identification by IEM.*

IEM can be used to identify viruses in 4 different ways: (a) Comparing the number of virus particles trapped by each of a number of antisera; (b) the clumping method of Milne and Luisoni (1975); (c) decorating the particles as described by Milne and Luisoni (1975); (d) the GLAD method developed during this research and as

described by Pares and Whitecross (in press). Each of these will now be examined.

Comparison of numbers of trapped virus particles. In section 5 I have established that counts of virus particles have a limited diagnostic value and an examination of the few published accounts of its use would tend to support this as shown in 5.7. My work has shown two principles of importance in using virus particle counts, (1) a single virus can be trapped on a range of antisera to determine its relationship to them, but more than one virus cannot be compared with a single antiserum as the starting virus concentrations would vary and the differences in counts would be due to this more than to serological relationships; (2) it is important to distinguish between counts reflecting true serological relationships and counts done under conditions to maximise differences between the numbers trapped by two antisera. I have examined this point and found that even when the differences are maximised the method is still not very reliable and subject to variations beyond the operator's control. It would seem that to maximise the differences a short trapping time is the best way. Antiserum coating could also do this but the attachment of IgG molecules to the grid is so quick that the times would most probably have to be less than 5 minutes and this could present problems if large numbers of grids were involved.

One of the main disadvantages in using particle counts is illustrated by the survey of tomato samples (section 7.3). Under the conditions chosen for trapping some samples had too few particles to gain any diagnostic information. If the conditions were changed in accordance with the findings in sections 3 and 5 to increase the virus density for these samples, then the other samples would have a

particle density that was too great to enable counting to be done. Even though a lot of information had been obtained on the IEM of the Tobamoviruses (sections 3-6), and even now having carried out the survey (section 7.3), it is still not possible to prescribe suitable conditions for a survey using particle counts diagnostically. The reason for this is the wide variation in particle concentration that can occur from one sample to another. Because nothing can be done to control this critical factor, there is no one set of trapping conditions that can be suggested.

Previous reports on the use of particle counts (see 5.7) have referred to samples of infected tissue that had been grown under glass-house conditions and had well developed symptoms. This enabled suitable conditions to be found and used to trap particles. Even under these controlled conditions there were indications of considerable variability and a lack of sensitive diagnostic separation.

Comparative particle counts, while having a limited diagnostic use, would probably be of greatest value when used to reflect the variations in virus concentration of the sample. Thus they could be used to determine virus concentrations in different host plants or possibly in different parts of the same host.

Clumping, as mentioned in 1.5, is also not a reliable way of diagnosing viruses, one of the main reasons being that, like virus particle counts, it is subject to variations due to the starting concentration of the virus preparation.

Decoration and GLAD. Under conditions of "controlled" samples or under field-sampling conditions the most efficient and sensitive method is decoration (with or without gold-labelling). It can be used under all conditions and is independent of the virus density

on the grid, as shown in published reports on "controlled" samples and in my work (sections 6 and 7) which constitute the first report of the use of IEM diagnostically on a range of field samples. The GLAD technique offers an increase in sensitivity over standard decoration in that it can demonstrate differences between isolates even when the decoration is too indistinct to be seen in the electron microscope.

8.2.3. *IEM and virus measurement.*

The measurement of virus particles is an important characteristic in the description of a virus and is used as part of the diagnostic process. It is of more importance, diagnostically, with rod-shaped viruses, in which the length can usually be used to allocate a virus to a particular virus group. Thus an expected diagnostic use of IEM might be to use the trapped particles for measurement, utilising the considerable increase in the density of particles on the grid, and its consequent saving in time when large numbers of particles are to be measured.

This aspect is discussed fully in section 4, and so this part of the work will only be mentioned briefly now. From published accounts the method appears to work well for isometric viruses and my observations on the Tymovirus group also supports this. When it comes to the rod-shaped Tobamoviruses the method does not work well, and my results establish that when particles are trapped onto a grid the population size distribution differs greatly from that produced by directly negatively staining a sap extract. Thus when using IEM for measurements of rod-shaped virus particles, care must be taken in the interpretation of the results.

8.3. Information obtained on Tobamoviruses

Even though this project was done to examine and evaluate the technique of IEM, using Tobamoviruses as a model, it is impossible to do so without finding information on the viruses themselves. Additionally part of the purpose of section 7 was to obtain information on the Tobamoviruses that occur in local orchid and tomato populations as this had not previously been done.

Tobamoviruses have been well studied serologically by various methods and the relationships between the 4 viruses used as models are given in the Materials and Methods (section 2). My results show the reliability of IEM in establishing serological relationships as all methods indicate that U2-TMV and ToMV are more closely related to each other than either is to U1-TMV and that ORSV is only distantly related to the other 3. Additionally the limited use of antiserum to CV4-TMV shows it is only distantly related to all 4 isolates used in my studies. This is in agreement with the results from other aspects of serology as summarised by Gibbs (1977), Fig. 2.

The Tobamoviruses in orchids and tomatoes have not previously been diagnosed with accuracy, other than to identify them as TMV-types. My results show that in orchids, all the isolates examined were of the ORSV type and all the IEM methods agreed with studies of host ranges. In this instance the two possible viruses were sufficiently distantly related serologically to enable the identification to be done easily by any of the IEM methods.

More variation was found among the virus isolates from Tomatoes. Because of the wide variation in concentration in the hosts, particle counting was not reliable and the best results were obtained by decoration and GLAD. The results demonstrate that all isolates

were more closely related to ToMV than to U1-TMV. In the 5 isolates studied in greatest detail there was a pronounced variation, one being much more closely related to ToMV than to U1-TMV, and 2 were clearly showing some relationship to U1-TMV but even so were still much closer to ToMV and were probably strains of it. This would tend to support findings throughout the world that a number of different strains of ToMV exist (Hollings and Huttinga, 1976).

One other point that was brought out in these studies concerns the fact that some members of the Tobamovirus group have a majority of particles 300 nm long, while others have been described as having bimodal population size distribution. This is discussed in much greater detail in section 4, in which it is noted that it is well established that two Tobamoviruses, Sunn-hemp mosaic and ORSV, have a bimodal population size distribution but are only described as having a unimodal population. It is suggested that descriptions of these two viruses have to be modified for the sake of uniformity, if for no other reason.

8.4. Concluding comments

As with any method based on serology, some idea of the identity of the virus or viruses being examined must be obtained before it can be used satisfactorily. When using IEM there appears to be no suitable standard method for use in all circumstances rather, the basic sources of variation must be understood and the IEM method should be varied to suit the situation being studied. This series of experiments represents the first detailed evaluation of the methods and the sources of variation. Given that the concentration of virus in the sample cannot be controlled, the most important factor in determining particle density on the grid has been shown to be the virus acquisition time.

The most reliable method to identify viruses on the grid appears to be the use of decoration characteristics or the GLAD variation. Counts of virus particles trapped are subject to variation from unknown factors even when using purified preparations of known concentration.

The development of the GLAD technique represents a considerable advance in the use of IEM for virus identification, especially in detecting distant relationships, and future development should enable its use in a greater variety of applications.

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PLATE 1

The use of antiserum-coated grids to increase the number of virus particles on a grid

Two viruses, ToMV (A, B) and ORSV (C, D) have been trapped onto a grid coated with homologous antiserum (A,C). In B the virus ToMV has been prepared from the same sap preparation as used for the trapping but has been mixed with negative stain and a drop dried on the surface of the grid. In D the virus ORSV has been trapped from the same sap preparation as used in C but the grid had been treated with antiserum to the dis

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ADDENDUM

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f the more sensitive

Preparations from IEM are also generally much cleaner, having much less contamination from other constituents of plant sap.

It can also be seen that coating grids with antiserum to a virus distantly related to the virus present in the sample does not result in many particles being trapped onto the grid, although in general it has been found in other work that coating grids with unrelated serum or normal serum is slightly better than using uncoated grids.

Bar on all micrographs ca. 500 nm.

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The use of antiserum-coated grids to increase the number of virus particles on a grid

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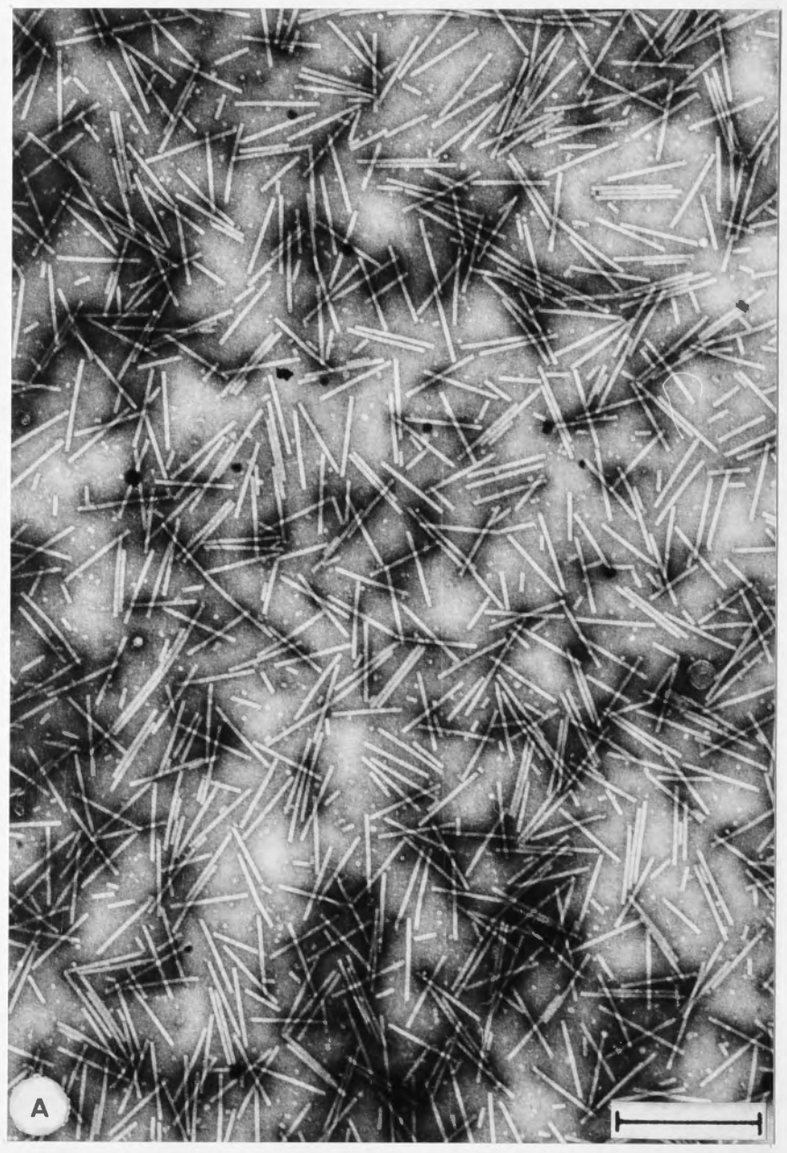
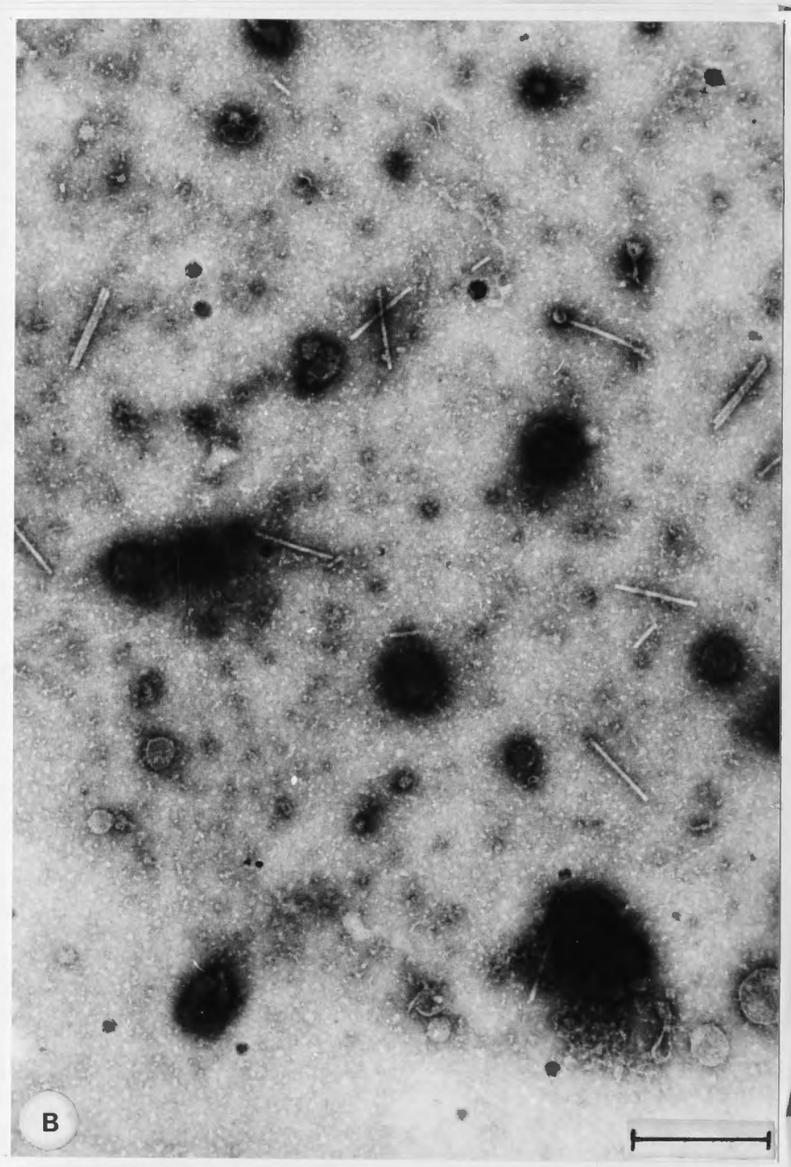
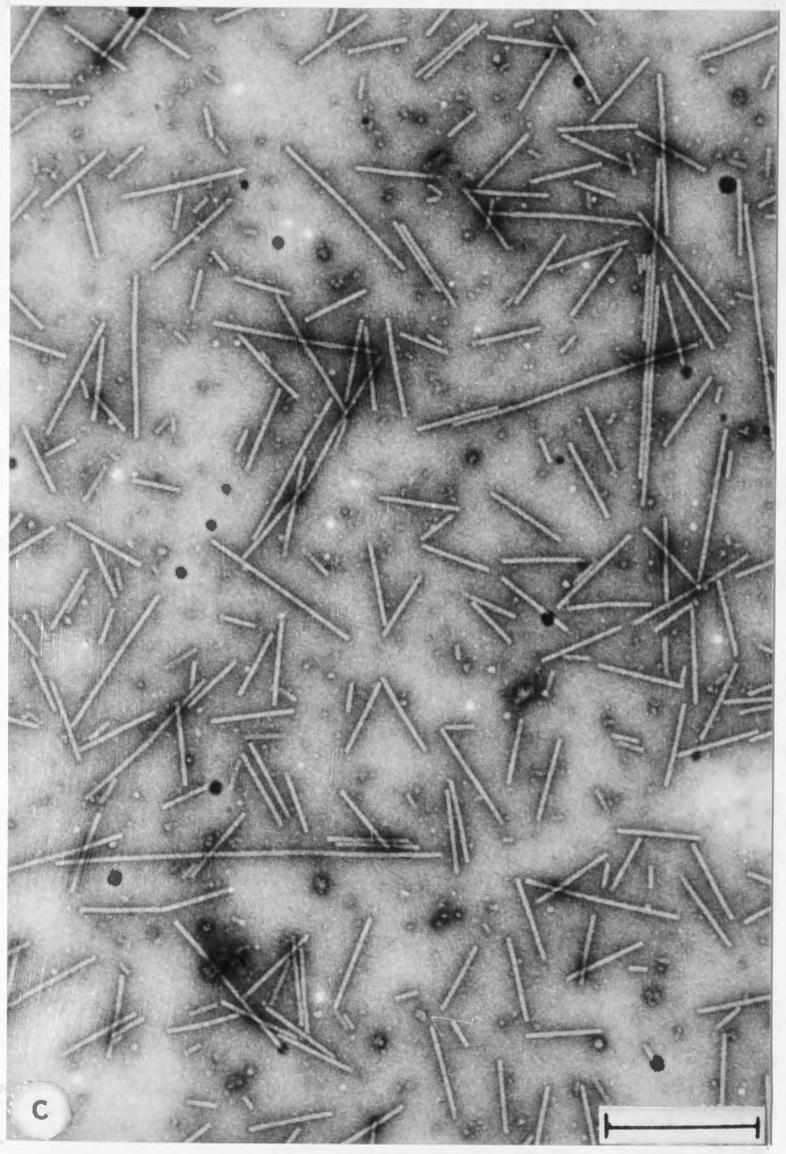
The plate shows the large increase in particle numbers that can be obtained by using IEM, resulting in the method being one of the more sensitive ways by which virus infections can be detected. Preparations from IEM are also generally much cleaner, having much less contamination from other constituents of plant sap.

It can also be seen that coating grids with antiserum to a virus distantly related to the virus present in the sample does not result in many particles being trapped onto the grid, although in general it has been found in other work that coating grids with unrelated serum or normal serum is slightly better than using uncoated grids.

Bar on all micrographs ca. 500 nm.







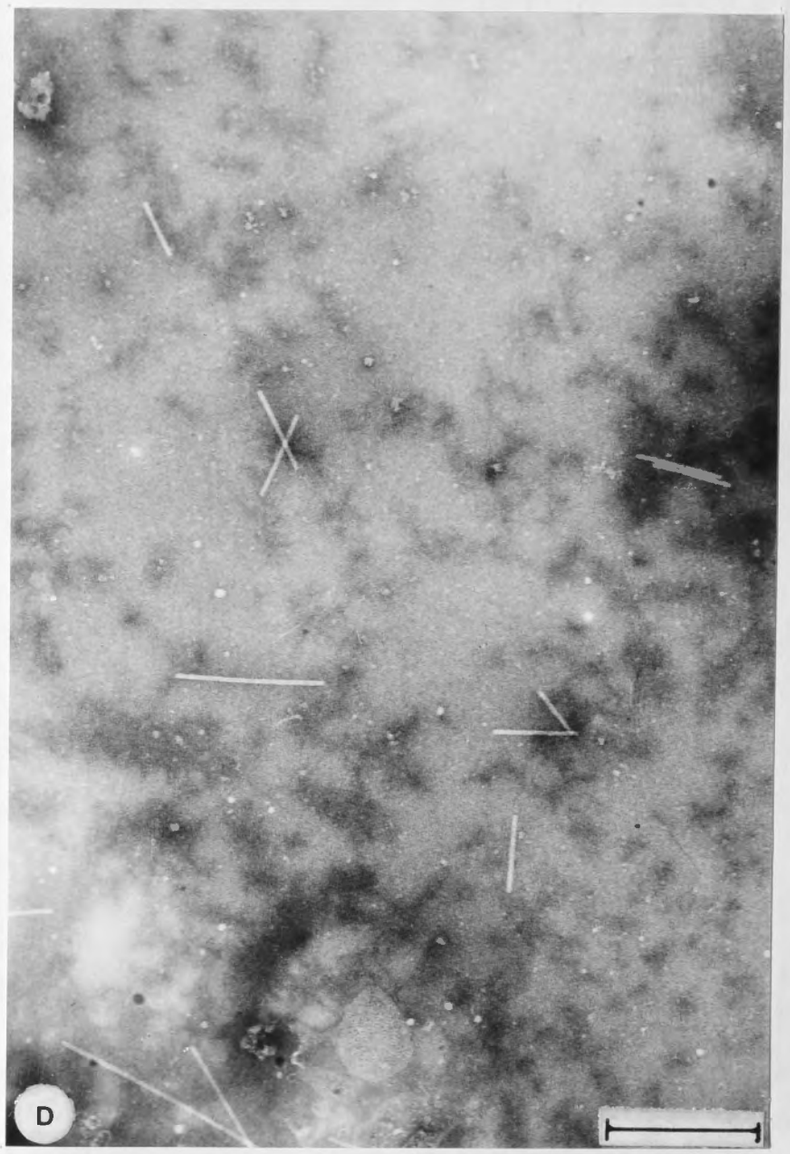


PLATE 1

PLATE 2

Decoration as a means of identifying viruses by IEM

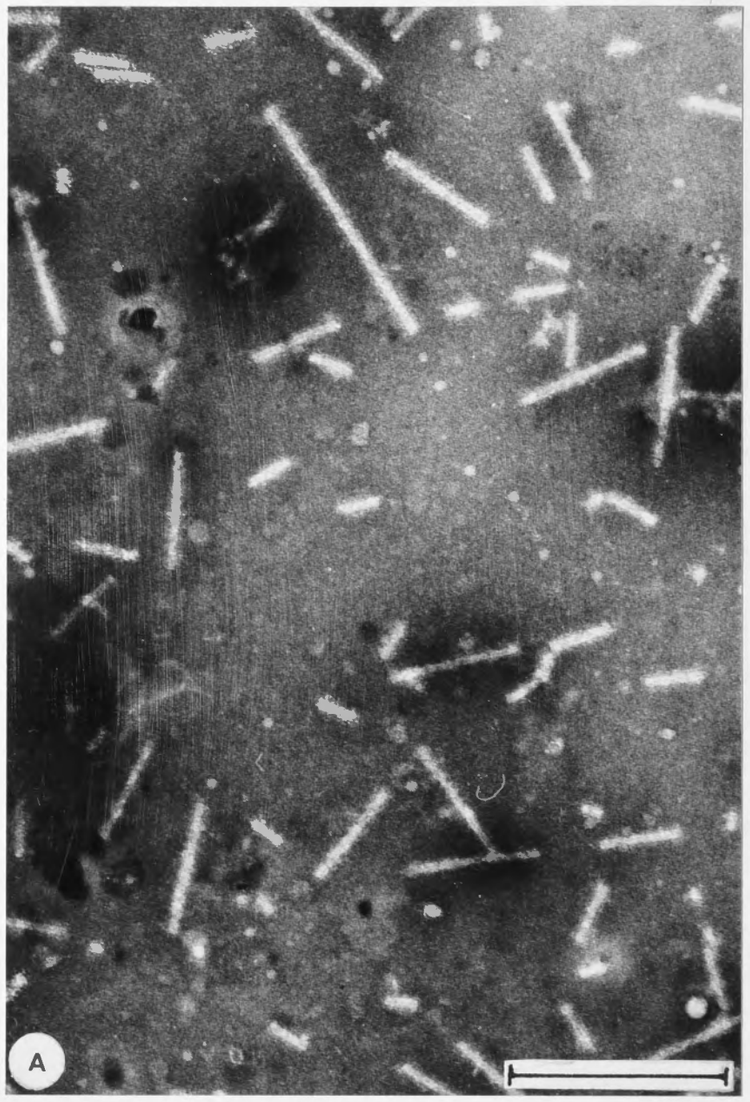
When virus particles on a grid are treated with antiserum any IgG molecules related to the antigenic determinants on the virus particles attach to the virus particles, making the outline of the virus particle less sharp. This is referred to in the literature as a "halo" of antibodies.

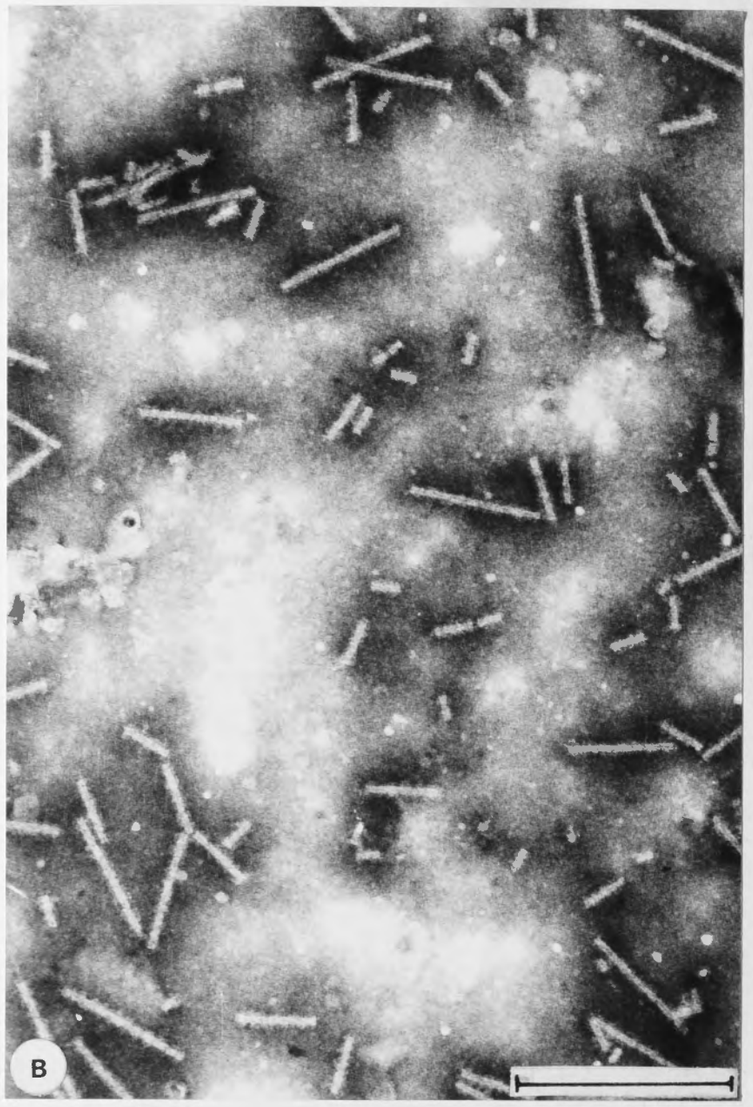
Some aspects of the use of this technique are illustrated in the 4 micrographs of a New South Wales isolate of ToMV treated with antiserum to the type isolate of ToMV.

When the decorating antiserum is used at a dilution of 1:16 (A) the decoration is heavy and the particles do not have a clear outline. There is no clear indication that the particles do have IgG molecules attached and the appearance could in fact be due to a faulty negatively stained preparation. When the antiserum is used at 1:32 the halo effect is not as pronounced (B) and it becomes more difficult to determine whether the particles are decorated or not.

The bottom two micrographs (C,D) illustrate the appearance of a preparation when only light decoration is present. The antiserum was used at 1:512. In such treatments the light decoration cannot readily be determined. This is similar to the effect when relationships between viruses are to be determined. Even though the antiserum may be distantly related to the virus, the light decoration cannot be seen, and the virus and antiserum would be considered unrelated.

The bar on A, B, D is ca. 500 nm and that on C is ca. 100 nm.





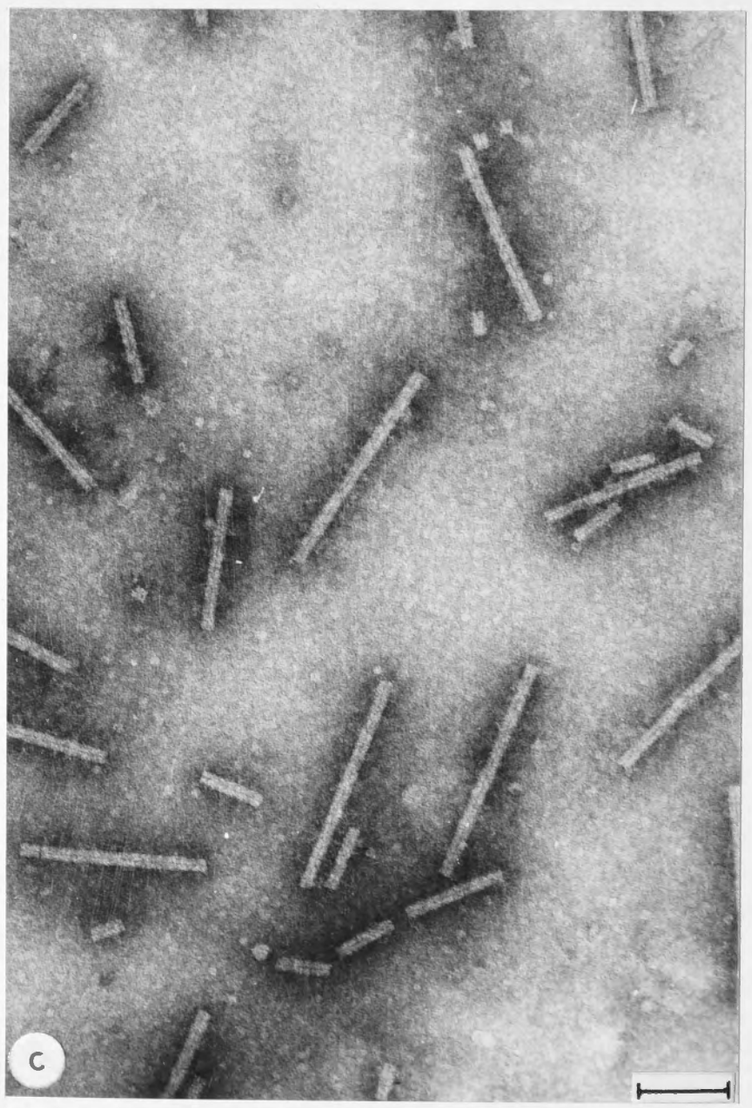


PLATE 2.

PLATE 3

Gold-labelled antibody decoration (GLAD)

The presence of light decoration can be determined with certainty when the IgG molecules are labelled with colloidal gold.

If the gold is applied after normal decoration with antibody at 1:1,000, the gold seems to have the same distribution with homologous antiserum as in A (U1-TMV, and a.U1-TMV) or a very distantly related one as in C (U1-TMV and a.CV4). However, when the same virus is decorated with the same two antisera that have been pre-mixed with colloidal gold (the GLAD method), the number of gold particles is much greater in the homologous treatment (B) than in the heterologous (D). This indicates that decoration does occur at an antiserum dilution of 1:1,000 and illustrates the fact that by using gold-labelling the level of decoration can be quantified, and can allow more distant relationships to be determined.

Fig.E is from the same treatment as D and only a very low level of gold is present, in this field only 2 can be seen (arrows). When the gold is premixed with normal serum the level of gold is very low and in most areas none is present (F).

Although in B the gold particles are generally near the virus particles, some appear to be not associated with the virus. When such preparations are examined at higher magnifications the gold can generally be seen associated with small pieces of the virus particles (G).

Figure H also illustrates the fact that in homologous labelling the gold particles are in general associated with areas of the grid in which virus particles are also present.

The bars on all micrographs except G are ca. 500 nm. The bar on Fig. G is ca. 100 nm.

PLATE 3

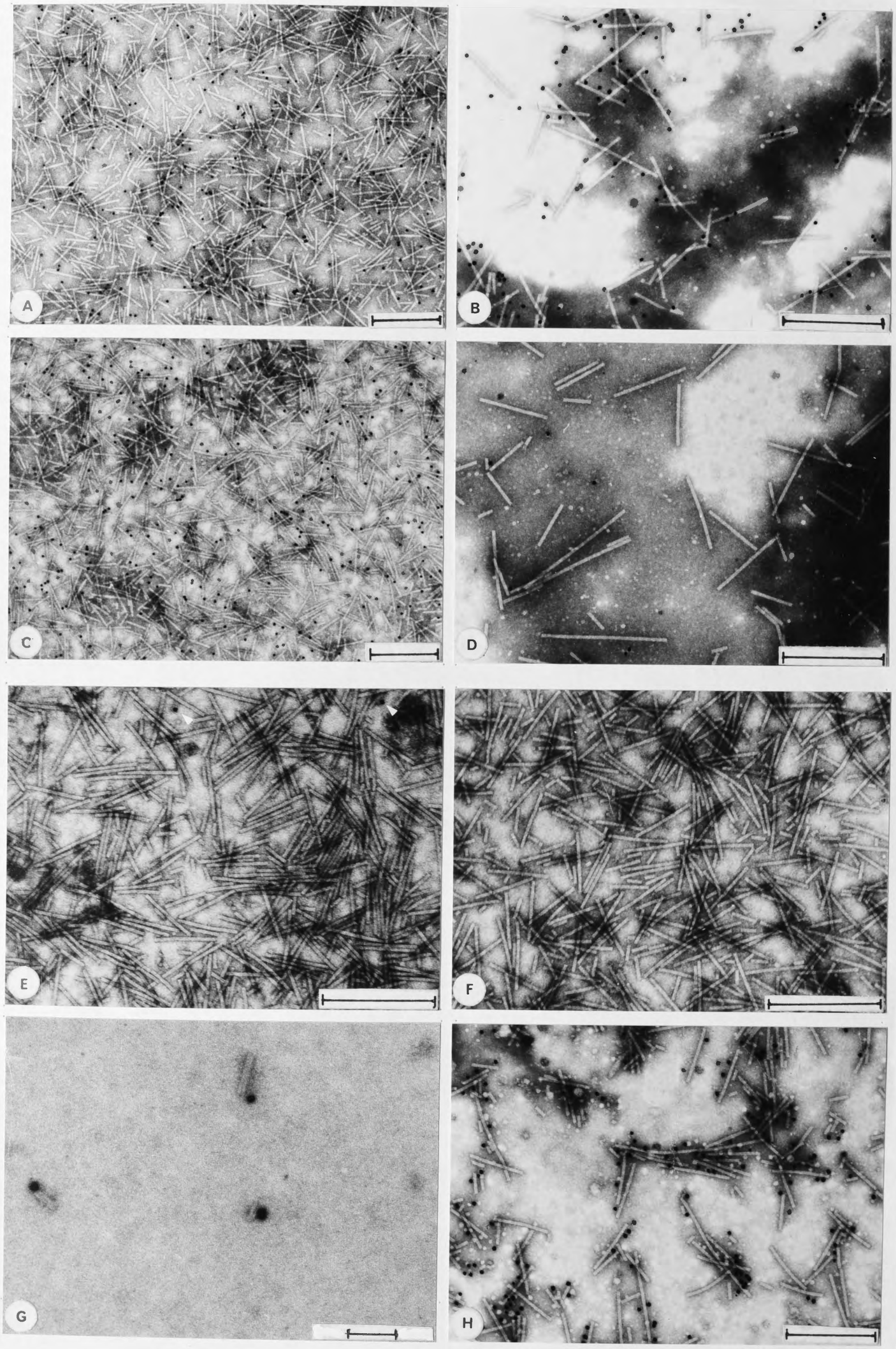


PLATE 3

This manuscript has been accepted for publication by the Journal of Immunological Methods and is due to appear in 1982.

The micrographs have not been attached to this copy of the manuscript as they are the same as those used to illustrate the use of the GLAD method in Section 6 of my thesis.

THE TECHNIQUE OF USING GOLD-LABELLED ANTIBODY DECORATION (GLAD) IN THE DIANGOSIS OF PLANT VIRUSES BY IMMUNO-ELECTRON MICROSCOPY

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SUMMARY

The diagnostic capabilities of immuno-electron microscopy have been developed by using antibodies labelled with colloidal gold-protein A. The method known as gold-labelled antibody decoration (GLAD) is described and results are given using the technique on a number of strains of tobacco mosaic virus.

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INTRODUCTION

The field of immuno-electron microscopy (IEM) combines some of the advantages of two major methods of virus detection and identification : electron microscopy and serology. While greatly increasing the ability to detect viruses in infected plant tissues it suffers from the disadvantage of not satisfactorily differentiating between strains of a virus. Milne and Luisoni (1975) found that once virus particles were on a grid antibodies could be added and they formed a "halo" around the virus particles with which they reacted. They termed this "decoration". The disadvantage of this method seems to be that light decoration is difficult to detect and decoration can sometimes appear to be similar to poor staining or other faults in preparation. The halo cannot be readily identified as being due to immuno-gamma-globulin (IgG) molecules.

In the detection of antigenic material in sections of tissue the antibodies have always been labelled to avoid any ambiguity and to enable better visualisation of any antigen - antibody reaction that may take place. Two main

types of electron-opaque markers have been used : ferritin and heavy metal colloids (of which gold has been the most successful).

The development and use of ferritin has been reviewed by Morgan (1972) who considered that the method had a number of drawbacks which he discusses. The two important ones are the fact that only a low percentage of antibody is labelled and that conjugation results in a considerable reduction in antibody titre.

The first use of colloidal gold as an antibody marker was by Faulk and Taylor (1971) and the method has been developed and modified by many workers since then for use in sectioned animal tissue. It has also been used on sectioned plant tissue (Craig and Millerd, 1981).

No one has satisfactorily adapted such antibody labelling techniques to "liquid preparations" such as plant virus particles on a grid. This report describes how antibody labelling with colloidal gold has been successfully applied to plant virus preparations on a grid. This development thus expands the usefulness of the labelled antibody technique and adds to the value of the IEM method not only as a very sensitive detection method but also as a good diagnostic tool.

MATERIALS AND METHODS

Five plant viruses, all belonging to the Tobamovirus group, were used : the U1 strain of tobacco mosaic virus (T_1 MV); the U2 strain (T_2 MV); tomato mosaic virus (ToMV); a local strain of ToMV (ToMV-N) and Odontoglossum ringspot virus (ORSV). The first three were kindly supplied by Dr A.J. Gibbs, Research School of Biological Sciences, Australian National University, Canberra. ToMV-N was

isolated from a glasshouse crop of tomatoes growing at Warriewood, N.S.W., and ORSV was isolated from an orchid at the Royal Botanic Gardens, Sydney.

The viruses were all increased in appropriate hosts. To purify the virus infected tissue was macerated in 0.05 M phosphate buffer containing 0.1% thyoglycollic acid at the rate of 4 ml per g tissue. The preparation was clarified with 8.3% n-butanol, given 2 cycles of differential centrifugation and then subjected to centrifugation through a 10-40% sucrose gradient. Antisera were produced in rabbits that were immunised by giving them one intravenous injection, followed by 2 intramuscular injections and then a final intravenous injection. Rabbits were bled 14 days after the last injection. Before use the antisera were diluted to 1:1000 in a buffer consisting of 1.59 g/l Na_2CO_3 and 2.93 g/l Na HCO_3, pH 9.6.

The gold-protein A (G/PA) was kindly supplied by Dr A. Millerd and Mr S. Craig, Division of Plant Industry, CSIRO, Canberra and was prepared as described by them (Craig and Millerd, 1981). It was diluted 1:2 in phosphate buffered saline, pH7.0, before use. The G/PA-antiserum conjugate was prepared by mixing 1 vol. of the dilute G/PA and 2 vols of the dilute antiserum.

Electron microscope grids were coated with parlodion/carbon. These grids were coated with unlabelled antibody and then placed onto drops of infected leaf tissue that had been ground in 0.06 M phosphate buffer pH 7.0. The grids were then washed in 20 drops of 0.03 M phosphate buffer and floated on 5-7µl drops of the G/PA - antiserum conjugate for 90 min. at room temperature. They were washed in 20 drops of buffer followed by 6 drops of 2% ammonium molybdate in distilled water, and dried by touching the edge of the grid on a piece of filter paper.

Electron microscopy was done using a Philips EM 301 at 60kV. Counts of G/PA particles were done, where necessary, by counting the number in 20 random binocular fields at a screen magnification of 5,500X.

Three experiments are to be reported here:

(1) A preliminary test of the technique to label particles of T MV with G/PA conjugated to 2 antisera closely related to the virus (a-T_1 MV and a-T_2 MV), one very distantly related antiserum to cucumber virus 4 (a-CV4) and normal serum (NS).

(2) Labelling of two closely related viruses (T_2 MV and ToMV) with each of the two corresponding antisera (a-T_2 MV and a-ToMV). In this experiment 5 grids were prepared for each of the 4 combinations, counts made on 10 spots per grid and a mean taken for each 5 grid combination.

(3) The application of the technique to a field situation in which a tobamovirus was extracted from an infected orchid plant and testing grids with G/PA conjugated to the antisera of the two tobamoviruses that have been recorded in orchids (a-T_1MV and a-ORSV).

RESULTS

Experiment 1. Preliminary Test of the Method

The two grids with the G/PA conjugated to the closely related antisera (a-T_1 MV and a-T_2 MV) had a good scattering of G/PA over them and there appeared to be some association of the G/PA with the virus. The two grids treated with G/PA - a-CV4TMV and G/PA-NS had hardly any G/PA present on them. A comparison between the reaction with a closely related antiserum and that with NS can be seen in Fig. 1.

Experiment 2. Comparative Labelling of Two Closely Related Viruses

The counts seemed to differentiate between the two viruses. For each virus - label combination the mean of the 50 counts was taken (10 counts per grid, 5 grids). These results are shown in Table. 1.

[SUGGESTED APPROXIMATE POSITION FOR TABLE 1]

Experiment 3. Application to a Field Situation

G/PA particles could be seen on grids labelled with both G/PA-a-T MV and G/PA-a-ORSV but in the latter case there was a considerable increase in the amount of G/PA over that seen on the grids using a-T_1MV in the conjugate and in addition to there being more G/PA present it appeared to have a greater association with the virus particles. Counts of the numbers of G/PA present gave a mean (for 10 counts) of 5 with a-T_1MV and 23 with a-ORSV, a ratio of about 1:4.5, thus giving a diagnosis of the virus as being ORSV. Subsequent tests have shown that the virus was ORSV.

DISCUSSION

Thomas (1980) compared four serological methods for the detection of rose viruses and found IEM more sensitive than any of the others including enzyme-linked immunosorbent assay (ELISA). Similar comparative sensitivities have been found by other workers with different viruses in different hosts. This has generally established the superiority of IEM as a method of virus detection, which is to be expected as IEM involves detection in the electron microscope and can thus get to the level of individual virus particles.

The two methods of ELISA and IEM are basically similar except that in ELISA the tests are done on a plastic surface and detection involves a reaction between an enzyme attached to decorating antibodies and a suitable substrate that changes colour. In spite of these similarities a major difference (and the great disadvantage with ELISA) is the ELISA possesses a very narrow strain specificity and enzyme conjugates prepared with antibodies against one virus strain give a negative reaction with other strains however closely related. This can be a serious disadvantage in diagnostic work and has led to investigations being made to overcome these faults (Van Regenmortel and Burckard, 1980).

For some reason, even though the two methods (ELISA and IEM) are similar there is no evidence of strain specificity being present with IEM.

However one of the disadvantages of IEM is that even though related strains can be picked up on an antibody coated grid there has to date been no quick, reliable method to detect which strain of virus is present on the grid.

For our experimental work we selected a range of tobamoviruses that showed differing degrees of serological relatedness. It is difficult to give exact figures for the degrees of relationships due to inherent variability in the figures due to individual rabbits and immunisation schedule. The range of such variations can be considerable and is discussed by Van Regenmortel (1975) in a paper in which he proposed a scheme of using a "serological differentiation index" (SDI). Using these figures Gibbs (1977) illustrated diagramatically the relationships of T_1MV, T_2MV, ToMV, and CV4. The serological relationship of ORSV to other tobamoviruses has been studied by Paul (1975).

Our results show that, using the GLAD technique, two distantly related viruses such as T MV and ORSV could easily be distinguished but the results with more closely related tobamoviruses were more equivocal. Such close relationships are probably more difficult to determine without utilising cross-absorbed antisera due to the reduction in antiserum titre that would occur after conjugation with G/PA. There is at present insufficient information on the combining ratios of the three principal reactants IgG, PA, and colloidal gold. There are no figures available on the number of PA molecules per gold particle and reports of combination of IgG : PA vary from 2:1 (Romano and Romano, 1977) to 4:1 (Roth, Bendayan and Orci, 1978). As a result the technique cannot yet be used for accurate quantitative work.

This point is illustrated by our results shown in Table 1 which indicate that each of the conjugated antisera satisfactorily differentiates the two viruses correctly but problems can arise when only one virus is present and a range of closely related, conjugated antisera are used to determine the identity of the virus. It may be possible to overcome these difficulties by future refinements of the technique, including the use of cross-absorbed antisera which is often necessary in other serological methods when closely related viruses are involved.

Thus for plant virus identification the use of IEM can greatly increase the sensitivity of detection, and GLAD can expand the most sensitive detection method into a good diagnostic tool.

ACKNOWLEDGEMENTS

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CAPTION FOR FIG. 1

The deposition of gold particles on a preparation of T_1MV treated with gold conjugated to the homologous antiserum (A) and conjugated to normal serum (B). The technique of virus preparation tends to break the particles up into small pieces some of which cannot be seen at this magnification but still attract gold-antiserum conjugate. (Mag. 102,800X). The bar on each micrograph represents 100nm.

Table 1. Counts of colloidal gold particles on 20 random spots per grid, 5 grids per treatment. Values given are the means of the 100 counts the standard error of the mean.

	Label. Colloidal gold conjugated with:	
Virus	a-T_2 MV	a-ToMV
T_2 MV	33.3 ± 1.39	12.2 ± 0.47
ToMV	20.0 ± 0.88	17.8 ± 1.01